NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

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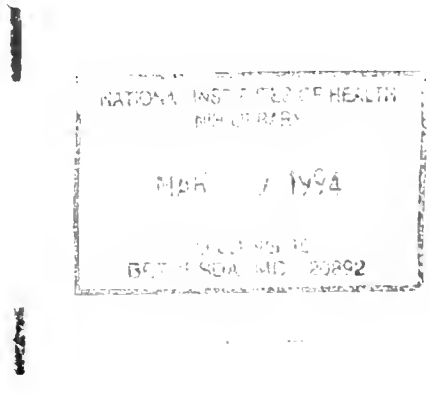

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National Institute of Diabetes and Digestive and Kidney Diseases

Investigators in the Diabetes Branch conduct clinical investigation and basic scientific research with special emphasis on understanding the mechanism of action of insulin and the causes of diabetes mellitus. Towards this end, a multi-disciplinary approach has been applied, involving techniques of molecular biology, cell biology, biochemistry, and clinical physiology. Other projects include studies of hormones and other messenger molecules important to the regulation of growth and development, especially growth hormone and insulin-like growth factors I and II. In addition, there is an active research program with respect to acromegaly, a clinical disease caused by the overproduction of growth hormone.

Insulin receptors

The insulin receptor is a heterotetrameric glycoprotein located on the cell surface. It is encoded by a single gene that encodes a precursor molecule. The precursor undergoes multiple post-translational processing steps including proteolytic cleavage to yield two different types of subunits: an α-subunit that is a tyrosine specific protein kinase. When insulin binds to the extracellular domain of the receptor, this activates the tyrosine kinase associated with the intracellular domain of the receptor. Activation of the tyrosine kinase plays a necessary role in mediating insulin action. Several projects in the Diabetes Branch pertain to various aspects of insulin receptor biosynthesis and insulin receptor function.

To study the regulation of the expression of the insulin receptor gene, the DNA in the 5' flanking region of the human and mouse insulin receptor genes have been cloned. Deletion analysis has been employed to identify a 70-base pair region of the gene that contains the majority of the promoter activity. In addition, a weak enhancer has been identified upstream of the promoter. When placed upstream of the minimal promoter, the enhancer increased expression of a CAT reporter gene when the reporter vector was transfected into HepG2 cells. In an electrophoretic mobility shift (EMS) assay, several retarded bands were observed using nuclear extracts from several cell types Although the enhancer does not contain an obvious Sp1 consensus sequence, an oligonucleotide containing a consensus Sp1 binding site competitively inhibited protein binding to the enhancer oligonucleotide. Furthermore, purified Sp1 protein binds to the enhancer oligonucleotide, although with lower affinity than it binds to a consensus Sp1 binding oligonucleotide. In addition, when HeLa cell nuclear extracts were preincubated with an anti-Sp1 antibody, an additional retarded band was observed due to the supershift of the Sp1/DNA complex. However, an oligonucleotide with a mutation that alters 2 bp in the enhancer sequence does not inhibit protein binding to the wild type enhancer oligonucleotide. These findings suggest that the proximal enhancer contains a novel sequence that binds Sp1 transcription factor, and this protein binding is likely to make a positive contribution to the expression of the gene.

Another potential cis-acting regulatory sequence has been mapped to the first intron of the human insulin receptor gene. When included in a reporter vector containing the CAT gene and stably expressed in 3T3-L1 cells, this sequence causes CAT expression to undergo a tenfold induction concomitant with differentiation of the 3T3-L1 cells into adipocytes. However, when the same expression vector was introduced into transgenic mice, this putative "adipocyte-specific enhancer sequence" in the first intron was not sufficient to direct significant expression of CAT in the mouse adipocytes. Instead, there was selective expression of CAT in the brain of transgenic mice derived from two separate founder animals. Apparently, 3T3-L1 cells are not a perfect in vitro model for this aspect of adipocyte physiology in vivo. Accordingly, we have developed the ability to transfect primary cultures of rat adipocytes. We have begun to investigate whether adipocytes retain their differentiated properties with respect to tissue-specific gene expression in tissue culture.

In another project, several steps involved in post-translational processing of the insulin receptor during biosynthesis are being investigated. In particular, N-linked glycosylation, O-linked glycosylation, and fatty acylation are being studied. The role of N-linked glycosylation is being investigated by site-directed mutagenesis to eliminate the N-linked glycosylation sites. By expression of cDNA's encoding these mutant receptors, it has been possible to investigate the functional role of N-linked glycosylation. When the first four glycosylation sites in the α-subunit of the receptor were abolished, this impaired intracellular transport of the receptor. The mutant receptor remained in the proreceptor form in the endoplasmic reticulum, but was not transported efficiently to the plasma membrane. When each of the four sites were mutated individually, the defect in intracellular transport was less severe. The defect introduced by simultaneous mutation of sites 1 and 2 was less severe than the defect caused by mutation of sites 3 and 4. These data support the hypothesis that N-linked oligosaccharide contributes to the forces that drive the folding of the proreceptor into its normal conformation.

The role of the tyrosine kinase in mediating insulin action is also being investigated. A 120 kilodalton glycoprotein in rat liver membranes has been identified to serve as a substrate for phosphorylation by the insulin receptor. This glycoprotein has been immunoaffinity purified, a partial amino acid sequence has been determined, and a cDNA encoding this glycoprotein has been obtained. This protein substrate, pp120, appears to be identical to a previously identified ecto-ATPase enzyme that has the property of hydrolyzing ATP and GTP. The effect of phosphorylation to regulate the enzymatic activity of this protein is presently being investigated. By expressing the cDNA through transfection, it should be possible to determine the physiological role of this protein, and what role the protein may have in mediating the effect of insulin upon target cells.

In addition, the gene encoding rat pp120/ecto-ATPase has been cloned. It contains nine exons and spans approximately 15,000 b.p. of DNA. Exon 7 undergoes variable splicing yielding two isoforms of mRNA. The isoform lacking

exon 7 encodes a short form of the protein in which the cytosolic domain is truncated to only 10 amino acids. This short isoform lacks the phosphorylation sites that are present in the long isoform of the protein.

In addition, as part of a long term collaboration with scientists at the University of Geneva, studies are underway to explore the mechanism and significance of receptor-mediated endocytosis, receptor internalization, and recycling. It has been shown that tyrosine phosphorylation is required for redistribution from the microvilli to clathrin coated pits, and as a result for ligand-stimulated endocytosis. These studies have been extended by investigating the effects of streptozotocin-induced diabetes in rats. Previous work had demonstrated that the diabetic state led to an impairment in internalization of ^{125}I-insulin by hepatocytes. Recent studies have suggested that the defect is more generalized; the diabetic state is associated with an impairment in receptor-mediated endocytosis of EGF as well as fluid-phase endocytosis. It is possible that this generalized defect may play a role in the pathogenesis of diabetic complications -- e.g., by impairing receptor-mediated endocytosis of low density lipoproteins and thereby causing hypercholesterolemia.

Considerable progress has been made in identifying mutations in the insulin receptor gene, and elucidating the role of this type of genetic defect in causing human disease. Multiple different mutations have been identified in the insulin receptor gene. These fall into five classes: class 1, mutations that inhibit insulin receptor biosynthesis, frequently by decreasing levels of insulin receptor mRNA; class 2, mutations that impair the transport of mutant receptors to the cell surface; class 3, mutations that decrease the affinity with which insulin is bound to the receptor; class 4, mutations that impair tyrosine kinase activity; class 5, mutations that accelerate receptor degradation, apparently by inhibiting recycling of internalized receptors back to the plasma membrane.

Recently, it has become increasingly apparent that this classification scheme is oversimplified. For example, we have identified a mutation in the tyrosine kinase domain of the receptor (substitution of Glu for Ala-1135). This mutation impairs tyrosine kinase activity (class 4), as predicted by the fact that it is located in a conserved sequence in the catalytic loop of the enzyme. However, despite its location in the intracellular portion of the receptor, the receptor also impairs proteolytic cleavage of the extracellular domain of the proreceptor into two subunits (class 2). Similarly, we have identified a mutation in the extracellular domain (substitution of Val for Phe-382) that impairs transport of the receptor through the endoplasmic reticulum and Golgi to the plasma membrane (class 2). In addition, this mutation inhibits the ability of insulin binding to induce a conformational change in the intracellular domain, thereby inhibiting the activitation of receptor tyrosine kinase (class 4).

One of the distinctive characteristics of mutations in the tyrosine kinase domain of the insulin receptor is that they cause insulin resistance in a dominant

fashion. It has been proposed that formation of hybrid dimers between normal and mutant receptors may explain the dominant negative effect of these mutations. To investigate this mechanism, we expressed two types of human insulin receptors in NIH-3T3 cells: wild type and the tyrosine kinase-deficient Ile^{1153}-mutant. To distinguish the two types of receptors, 43 amino acids were deleted from the C-terminus of the wild type receptor (Δ43-truncation). If mutant and wild type receptors assemble in a random fashion, 50% of the receptors would be hybrid oligomers ($\alpha_2\beta\beta_{mut}$). However, $\alpha_2\beta\beta_{mut}$ hybrids were undetectable. Nevertheless, insulin stimulated transphosphorylation of the kinase-deficient Ile^{1153}-mutant receptor by the kinase competent Δ43-receptors via an intermolecular mechanism in co-transfected cells. Furthermore, transphosphorylation of the Ile^{1153}-mutant receptor is sufficient to trigger insulin-stimulated endocytosis. Despite the absence of $\alpha_2\beta\beta_{mut}$ hybrids, expression of the Ile^{1153}-mutant receptor inhibited the ability of the Δ43-truncated receptor to mediate insulin-stimulated phosphorylation of insulin receptor substrate-1 (IRS-1). Evidence was obtained to support the hypothesis that the Ile^{1153}-mutant receptor retains the ability to bind IRS-1, and that sequestration of substrate may explain the dominant negative effect of the mutant receptor to inhibit phosphorylation of IRS-1.

Previously, we had identified several patients with two mutant alleles of the insulin receptor gene. In some cases, one of these alleles was a null allele that encoded a truncated fragment of the receptor that was predicted to lack all function. Nevertheless, there had been no report of a patient with two null alleles. Indeed, it had been suggested that homozygosity for a null allele might be lethal during embryonic development. However, during the past year we have identified two patients with leprechaunism who were homozygous for null alleles. In both cases, the parents were first cousins so that the children were homozygous by descent for a mutant allele -- in one case a total deletion of the insulin receptor gene, and in the other case a short deletion introducing a premature chain termination codon in the extracellular domain of the receptor. During the past year, we have made good progress in our efforts to establish a transgenic mouse model of mutations in the insulin receptor gene. We have inactivated one allele of the insulin receptor gene in embryonic stem cells, and have obtained chimeric mice. It is not yet known whether there will be germ line transmission of the mutant gene.

From a therapeutic point of view, we have attempted to treat several insulin resistant patients with insulin-like growth factor I (IGF-I). It had been proposed that IGF-I might act through the type 1 IGF receptor to elicit insulin-like actions in target cells such as skeletal muscle. However, in contrast to publications from several other investigators, IGF-I has had little if any effect upon glucose levels in our patients. These observations suggest that intact insulin receptors may somehow be required to enable the type 1 IGF receptors to elicit insulin-like actions in vivo.

Glucose Transport

The ability of insulin to promote glucose transport into target cells (e.g., skeletal muscle and adipose tissue) is among the most important actions of the hormone in vivo. Investigators in the Diabetes Branch were the first to provide a molecular explanation of this action of insulin. In the absence of insulin the GLUT-4 isoform of glucose transporter is localized primarily in intracellular vesicles. Insulin stimulates the recruitment of these vesicles to the plasma membrane. Investigations are currently underway to elucidate the detailed molecular mechanisms that underly this biological action of insulin.

The subcellular trafficking of tracer-tagged GLUT4 between the plasma membranes and low-density microsomes of rat adipose cells has been studied. Cell-surface GLUT4 have been initially tracer-tagged in the insulin-stimulated state with a [3H]-bis-mannose. The initial experiments show that insulin does not alter the half-time for GLUT4 endocytosis but instead increases the rate of exocytosis. Additional data suggest that the cells' entire complement of GLUT4 is involved in the recycling process. In order to obtain reliable kinetic constants for the two glucose transporter isoforms found in insulin responsive tissues, each transporter was expressed in Xenopus oocytes by the injection of mRNA encoding rat GLUT1 or GLUT4. The 3-0-methylglucose kinetic data indicate that, at low substrate concentrations, the catalytic efficiency of GLUT4 is significantly greater than GLUT1. Extrapolation to mammalian systems suggests that GLUT4 is responsible for virtually all of the hexose uptake in insulin responsive targets, particularly in the presence of hormones. In studies of the effects of K^+ depletion, we observed an inhibition of GLUT4 internalization which is entirely analogous to the effects on IGF-II/Man-6-P receptor cycling strongly suggesting the involvement of a coated pit mechanism in the recycling of GLUT4 transporters. An inactive conformation of GLUT4 has also been detected in plasma membranes from insulin-stimulated cells which is enhanced by K^+ depletion without a corresponding increase in transport activity suggesting a limit in the adipose cells' capacity to promote active GLUT4 transporters.

In addition, glucose transport is being studied in tissues where it is not primarily under the regulation of insulin. The incidence and severity of stroke is significantly higher in diabetic patients compared to the normal population and the subsequent prognosis poorer. A primary objective of our studies has been to determine whether a relationship exists between these observations and the mechanisms by which the central nervous system (CNS) disposes of glucose. To this end it was first important to determine which glucose transporter isoform is responsible for mediating the transport of glucose into the various cells that constitute the CNS and what effect stresses such as diabetes, hypoxia, and ischemia have on the expression of these transporters. Until recently only one isoform of glucose transporter was identified in brain, the high molecular weight (Mw) 55Da GLUT1 which is responsible for transporting glucose across the endothelial cells

that make up the blood brain barrier. We have subsequently described a second, small, less glycosylated, form of GLUT1 (45kDa) which is detected in a vessel-free, neuronal/glial fraction. Based on cell culture studies and immunohistochemistry, the 46kDa GLUT1 isoform is most likely to reside in non-neuronal cells, i.e. astroglia. This isoform has been detected in primary neuronal cultures but at levels that are 1/10 that of the predominant neuronal glucose transporter, GLUT3. In addition, we have discovered the presence of another glucose transporter isoform, GLUT5, in microglia of the CNS of both humans and rats, as detected by immunohistochemistry.

We have investigated the effects of diabetes on glucose transporter expression in two distinct rat models. In the streptozotozin-treated rat, acute diabetes is associated with a rapid (by day 3) increase (20%) in the expression of the neuronal GLUT3 glucose transporter protein in the neurohypophysis which persists over a 4 week period. Concomitant with this increase, the low Mw GLUT1 isoform is decreased by 20% on day 3 is 53% decreased by 4 weeks. This represents the first stress-related alteration in GLUT3 expression which presumably reflects the increased vasopressin secretory activity induced by the dehydrating effects of the acute untreated diabetes. The underlying mechanism for these changes are currently under investigation.

The second model of diabetes we have studied is the BB-Wistar rat which spontaneously develops diabetes. We have looked at the long term effects of diabetes and episodic hypoglycemia in both control and diabetic animals on the expression of GLUT1 and GLUT3 glucose transporters in various brain regions. Glucose transporter levels were measured in the various regions by Western blot analysis and quantitated by phosphorimage analysis. The recurrent hypoglycemia had no major effect on either GLUT1 or GLUT3 expression in either control or diabetic animals. Diabetes elicited a significant increase in the concentration of GLUT1 (18-63%) in temporal and frontal cortex, hippocampus, brain stem and hypothalamus but a decrease in cerebellum (10%) and pineal glands (40%). Increase in GLUT3 concentration were observed (18-38%) only in hippocampus, neurohypophysis, and brain stem. Thus contrary to our earlier observations in peripheral tissue where diabetes promotes a decrease in glucose transporter expression, in the CNS there is region specific up-regulation of both GLUT1 and GLUT3 glucose transporter.

Insulin-like growth factors

Mammalian IGF-I genes are composed of at least 6 exons. The mature peptide is encoded by exons 3 and 4. As a result of variable splicing and the use of alternate polyadenylation sites, there are multiple species of IGF-I mRNA ranging in size from 0.8 - 7.5 kb in length. Transcripts containing exon 2 are more sensitive to regulation by growth hormone. Transcripts containing exon 1 are expressed ubiquitously in all tissues, but are less sensitive to regulation by growth hormone.

The mammalian IGF-I genes are composed of at least 6 exons. Exons 1 and 2 each contain multiple transcription start sites, and encode different 5'-untranslated regions (5'-UTR). Exons 3 and 4 encode the mature peptide, whereas exons 5 and 6 encode different E peptides and the variable 3'-UTR. This complexity in the gene probably relates to the widespread expression of IGF-I in every tissue of the body, yet with differing developmental expression and different tissue-specitic functions. More primitive vertebrates such as the fish have a slightly less complex gene. The salmon IGF-I gene contains only one leader exon; however, some complexity exists in the splicing of the E peptide-encoding exons where a number of alternatives exist. Furthermore, fish have a larger number of chromosomes in their genome and therefore, have two non-allelic IGF-I genes.

Studies on the expression of the IGF-I gene have revealed that, in the rat, IGF-I is expressed in a developmental- and tissue-specific manner and that this is reflected by alternative usage of the leader exons. The putative promoter region of leader exon 1 has multiple transcription start sites with no TATA/CAAT elements, whereas, that for leader exon 2 has a single major transcription start site with TATA and CAAT elements situated in the correct positions in the 5'-flanking region. Both promoter regions were placed upstream of a luciferase reporter gene and transfected into CHO cells, where basal activity was observed. In addition to differential promoter usage and alternative transcription start site, the IGF-I gene encodes elements which may control translation. Thus, depending on which promoter is used, different translation start sites will encode leader peptide sequences of varying length. Furthermore, alternate usage of exon 5 or 6 results in different E peptides; one of which has N-linked glycosylation sites while the other does not. Thus the study of the complexity of the mammalian IGF-I gene with potential transcription, translation and post-translational differences may help in the understanding of this ubiquitously expressed important hormone.

Insulin-like growth factor receptors

The biological effects of IGF-I are mediated through a cell surface receptor, a heterotetromeric glycoprotein that is homologous to the insulin receptor. A cDNA encoding a portion of the IGF-I receptor has been cloned from rat granulosa cells. A portion of the 5'flanking region of the IGF-I receptor gene has been cloned from the rat.

To elucidate those factors involved in the regulation of the IGF-I receptor, we have characterized the proximal promoter of the human and rat IGF-I receptor. The promoter lacks the classic TATA-CAAT element upstream of its single transcription start site which itself is encompassed by an "initiator (INR) sequence which apparently directs transcription from the single site in the absence of a TATA box. Another unusual feature is the relative long (~1-Kb) 5' untranslated region which, similar to the 5' flanking region, is relatively GC rich. Multiple potential binding sites ("cis-elements") for transcription factors are found, including those for SPl, AP2, ETF, GCF and Wilms' tumor (WT/EGR). Co-transfection of

an SP1 expression vector with the proximal promoter upstream of a luciferase reporter gene (±400bp of 5'-flanking region) in Schneider (Drosophila) cells revealed that SP1 enhances basal promoter activity 60-80 fold. At least 10 potential SP1 binding sites are found in the region of the transcription start site, some in the immediate 5' flanking region and the rest in the 5'UTR. The level of response to SP1 is also dependent on the number of SP1 sites included in the promoter plasmid. Co-transfection of the IGF-I receptor promoter constructs with a plasmid encoding the Wilm's tumor (WT-1) gene product inhibited promoter activity. This effect was also dependent on the number of potential WT1 sites, of which there are more than 10 in the promoter region. DNAse I footprinting and gel mobility shift assays were used to complement the co-transfection experiments. Gene fragments containing one or more WT-1 sites were retarded on the gel in the presence of WT protein; those without WT sites were not. These sites were also confirmed in DNAse I footprinting experiments. Wilm's tumor tissues were compared to normal kidney tissue and the level of IGF-I receptor mRNA was higher in Wilm's tumor tissue, and was inversely correlated with the level of WT mRNA. These studies strongly suggested that the WT product negatively regulates the IGF-I receptor and therefore the IGF-I receptor may play an important role in the initiation and propagation of Wilm's tumor during the initial blastemal stage of kidney development.

In addition, studies have been initiated to define structure-function relationships of the IGF-I receptor. Toward that end, the key lysine in the ATP-binding site was mutated to an alanine. This mutation inhibited receptor tyrosine kinase activity, and also inhibited the ability of the receptor to mediate the effects of IGF-I to stimulate DNA synthesis, 2-deoxyglucose uptake, and phosphatidyl inositol-3-kinase activity. These observations suggest that receptor tyrosine kinase activity is required for the receptor to mediate biological activity. In addition, mutations have been made to eliminate the three tyrosines in the major autophosphorylation site (Tyr -1131, -1135, and -1136). This triple mutant was also impaired in its ability to mediate IGF-I action. Another mutant was constructed, in which there was a deletion of 99 amino acids at the C-terminus of the receptor. Although the C-terminal deletion decreased autophosphorylation, it did not inhibit phosphorylation of the endogenous protein substrate IRS-1, nor did it impair the mitogenic action of IGF-I.

Insulin-receptor related receptor

The insulin receptor related receptor (IRR) is an orphan receptor that is homologous to the receptors for insulin and IGF-I. The gene encoding this protein was originally identified by low stringency hybridization of Southern blots of human and guinea pig genomic DNA probed with fragments of insulin receptor cDNA. Predictions of the pattern of splicing of the 22 exons in the IRR gene were based upon analogy to the insulin receptor gene. To test these predictions, we have cloned IRR cDNA from a human kidney cDNA library. The deduced amino acid sequence of our cDNA was identical to the published amino acid sequence of

human IRR with one exception. There was an insertion of 24 base pairs (encoding 8 amino acid residues) between exons 13 and 14. This insertion was caused by use of an alternative splice acceptor site in the 3'-portion of intron 13. Interestingly, this alternative splicing occurs at a position where there is also alternative splicing of the IGF-I receptor mRNA. The full length cDNA encoding human IRR has been expressed by transfection in NIH-3T3 cells. This will enable us to study the structure and function of the IRR. In addition, studies are underway to identify the natural ligand for the IRR.

As one approach to elucidating the function of this receptor, we have undertaken to inactivate the IRR gene by homologous recombination. As a first step toward that goal, we have cloned genomic DNA encoding the murine IRR. The gene contains 22 exons, and spans >20 kbp of genomic DNA. The availability of the cloned murine IRR gene has enabled us to construct a targeting vector to inactivate the IRR gene in embryonic stem cells using the technique of "positive-negative selection". These studies are currently underway.

Using probes derived from the sequence of the murine IRR gene, we screened a murine kidney cDNA library and obtained overlapping cDNA clones spanning a portion of the cDNA (nucleotides 851-3270). The remainder of the murine kidney IRR sequence was obtained by using reverse transcriptase (RT) and thermostable DNA polymerase to amplify IRR cDNA by RT-PCR (using protocols for the rapid amplification of 5'- and 3'-ends of cDNA). The deduced amino acid sequence of murine IRR was 89% identical to the deduced sequence of human IRR, and 87% identical to the deduced sequence of guinea pig IRR. In addition, at least 3 splicing variants were identified in IRR mRNA from murine kidney. In the major species, all 22 exons are spliced together with a 3900 nucleotide open reading frame as predicted in previous publications about the IRR. In the second species, an alternative splice acceptor site is used in exon 2 such that 149 bp are deleted from the 5'-end of exon 2. In the third species, an alternative splice acceptor site is used in intron 1 such that 68 bp of sequence are inserted between exons 1 and 2. The latter two splicing variants lead to frameshifts and premature chain termination codons within the exon 2 sequence.

Growth hormone and acromegaly

A long term follow-up study of patients with acromegaly has been conducted in the Diabetes Branch over the past 25 years. Several modes of therapy have been employed, including radiation therapy, trans-sphenoidal hypophysectomy, and more recently treatment with drugs such as bromocryptine and somatostatin analogs. Recent studies have demonstrated that somatostatin analogs can be extremely useful in lowering growth hormone levels in patients with acromegaly. Unfortunately, somatostatin analogs have a side effect of causing the patients to develop thickened bile. Because of the previous observation that patients with somatostatin secreting tumors have a high prevalence of gallstones, it seemed important to determine how frequently treatment with somatostatin analogs would

also cause this complication. Of patients who have received long-term treatment with the long-acting somatostatin analog, 70% of the patients with acromegaly have developed gallbladder sludge and 20% have developed gallstones. Patients currently enrolled in this study who have persistent sludge or gallstones will be treated with ursodeoxycholate, a drug with the potential to prevent or to reverse the formation of sludge and gallstones. Ursodeoxycholate has dissolved gallstones in 3 out of 5 patients, and has dissolved sludge in 1 out of 3 patients.

Quantitation of insulin actin *in vivo*

Since insulin resistance and impaired glucose effectiveness may play a role in the pathogenesis of NIDDM, quantitative assessment of these properties is of interest. Recently, a minimal model approach involving mathematical modeling and computer simulation has been used to estimate both insulin sensitivity and glucose effectiveness from the results of a single frequently sampled intravenous glucose tolerance test (FSIVGTT). The equations of the minimal model describe changes in plasma glucose concentration as functions of insulin and glucose concentrations. A computer program identifies model parameters that generate a best fit to insulin and glucose data obtained during the FSIVGTT. Thus, the minimal model is able to estimate the relative contributions of insulin and glucose to glucose tolerance. We have used the minimal model to generate specific predictions of both insulin-dependent and insulin-independent glucose metabolism under a variety of conditions. We performed experiments in subjects with insulin dependent diabetes mellitus and in normal subjects to test these predictions. By comparing model predictions with experimental results, we were able to demonstrate that the minimal model underestimates the contributions of insulin and overestimates the contribution of glucose to glucose metabolism. Studies are presently underway to confirm these results and to understand the origin of the discordance between minimal model predictions and experimental results. The data that we collect may allow us to formulate a more accurate and useful mathematical model.

Regulation of gene expression

One of the central questions in developmental biology relates to how genes are regulated in a tissue-specific manner. For example, the insulin gene is expressed at high level in the pancreatic beta cell while the gene is essentially shut off in other tissues. In order to define the molecular mechanisms involved in this type of regulation, the beta globin locus has been used as a model system. Thus far, the chicken beta globin locus has been analyzed using both transient expression in cultured cells and also expression in transgenic mice. These studies have identified multiple regulatory elements including a locus control region that allows for position-independent copy number-dependent expression of the gene.

We previously demonstrated that the chicken β^A-globin gene and its 3' enhancer contain information sufficient to guarantee copy-number dependent

expression in transgenic mice, indepe
ndent of the site of transgene integration (this property defines a locus control region, LCR). To study the way in which an enhancer/locus control region activates chromatin, we examined transgenic mice carrying various combinations of the chicken β^A-globin gene coding region, promoter and 3'enhancer/LCR. We compared lines carrying only the coding region and enhancer/LCR (E) and only the coding region and promoter (P) with those containing all three elements (PE). We examined chromatin activation by monitoring formation of erythroid-specific DNase I hypersensitive sites and transgene transcription. All of the PE lines, none of the P lines and some (3 of 6) E lines showed hypersensitivity. While all of the PE mice transcribed the transgene in a copy number-dependent manner, none of the P mice expressed their transgene. The two E lines with strong hypersensitive sites had RNA complementary to the transgene, presumably initiating from an adjacent adventitious mouse promoter. These results support a 'mutual interaction' model for the mechanism of chromatin opening by LCRs in which an enhancer/LCR and a promoter must cooperate to generate open chromatin. The data are not consistent with a 'dominant enhancer' model in which the enhancer/LCR can open chromatin autonomously.

As another approach to understanding the organization of the chicken ß-globin cluster, we have undertaken its sequencing. Analysis of the sequence suggested that duplication of the ancestor ß-globin gene produced the ϵ and the $\rho/\beta^H/\beta^A$ ancestor genes, which duplicated to form the p and the β^H/β^A ancestor genes and finally the β^H- and β^A-globins. Four gene conversions can be documented: β^A to β^H, ϵ to β^H and ρ/ϵ (twice). An L1-like repetitive element, CR1, accounts for 16% of the cluster which is a surprisingly large fraction and suggests that the cluster is a hot spot for CR1 retrotransposition.

Honors awarded to present and former members of Diabetes Branch:

In addition to the scientific research carried out in the Diabetes Branch, one of our most important functions is to train young investigators. The outstanding accomplishments of the Diabetes Branch in this regard have been recognized by several awards during the past year. Dr. Efrat Wertheimer (a Visiting Fellow in the Diabetes Branch) received two awards for her work: the Nicholls Institute New Investigator Award bestowed by The Endocrine Society, and the Henry Christian Award of The American Federation for Clinical Research. The latter award was in recognition of the fact that Dr. Wertheimer's abstract received the highest rating among all the abstracts submitted in the subspecialty of Endocrinology and Metabolism. Dr. Takashi Kadowaki (a former Visiting Fellow, 1986-1989) received two Young Investigator Awards -- from the Japan Diabetes Association and The Japanese Endocrine Society. Dr. Jesse Roth (previously, Chief of the Diabetes Branch) was awarded the Albert Renold Award of the American Diabetes Association in recognition of his outstanding accomplishments as a mentor of young investigators. Finally, Dr. C. Ronald Kahn (who was trained as a Clinical Associate in the Diabetes Branch and eventually rose to become a Section Chief) received

the Banting Award, the highest scientific award of the American Diabetes Association.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK 47001-11

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Phosphorylation of Insulin and IGF-I Receptors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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LAB/BRANCH

Digestive Diseases Branch

SECTION

Diabetes Branch

INSTITUTE AND LOCATION

Section on Gastroenterology

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

IGF-I receptors regulate the growth-promoting and differentiative effects of IGF-I and IGF-II. The IGF-I receptor, though endcoded by a seperate gene, is structurally and functionally related to the insulin receptor, both being transmembrane tyrosine kinases. To investigate structure/function relationships in the IGF-I receptor, we have used a number of different techniques, including (A) isolation and genes, (B) mutagenesis of the tyrosine kinase domain of the human IGF-receptor and (C) analysis of post-receptors events. These studies should contribute to our understanding of the role of IGFs and the IGF receptor in health and disease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK47005-21 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of insulin receptors in circulating cells in man

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I.	P. Gorden	Director	NIDDK
Others:	C. Hendricks	Biol. Lab. Tech.	DB, NIDDK
	E. Collier	Medical Officer	DB, NIDDK
	P. Roach	Clinical Associate	DB, NIDDK
	P. Formisano	Special Volunteer	DB, NIDDK
	S.I. Taylor	Chief	DB, NIDDK
	Y. Zick	Visiting Scientist	DB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The present work continues prior investigation of insulin receptors on circulating cells in patients with insulin resistance and diabetes mellitus. Insulin receptors are evaluated for their ability to bind insulin and to act as tyrosine-specific protein kinases. We have specifically studied two individual patients: one with a Type A form of insulin resistance that binds insulin normally but has reduced tyrosine-kinase activity, and the second, a patient with a Rabson-Mendenhall Syndrome, who has a severe defect in insulin binding.

In addition, we have continued to study patients with autoantibodies to the insulin receptor associated with insulin resistance.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47007 17 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Antibodies to Receptors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I. S. Taylor Chief, DB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Diabetes Branch

SECTION

Receptors & Hormone Action

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

I N A C T I V E

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 DK 47009-06 DB

PERIOD COVERED
October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Positron Emission Tomography/NMR Spectroscopy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I.	R. Eastman	Director	DDEMD,NIDDK
Others:	S. Taylor	Chief	DB,NIDDK
	D. LeRoith	Section Chief	DB,NIDDK
	C. Cochran	RN	CC
	B. Koller	Senior staff Fellow	DB, NIDDK
	M. Skarulis	Clinical Investigator	NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Two protocols have been developed and approved (91-DK-133) and 91-DK-97) to use positron emission tomography (PET) and nuclear magnetic resonance spectroscopy (NMR) to study insulin and IGF-1 action in patients with insulin resistance. Due to technical problems, the NMR investigation in 91-DK-133 was discontinued at the time of the last protocol review. In the last year no patients were studied using this methodology. A protocol is being developed to study pre-diabetic Pima indians which will include this technique.

One patient with IGF-II induced hypoglycemia from adrenocortical carcinoma was studied using PET in the last year.

91-DK-97 has since been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47018-15 DB

PERIOD COVERED

October 1, 1992 - September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cellular Hormone-Like Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I.'s:	D. LeRoith	Section Chief	DB/NIDDK
	C.T. Roberts, Jr.	Research Biol.	DB/NIDDK
Others:	M. Adamo	Staff Fellow	DB/NIDDK
	S. Shemer	Guest Worker	DB/NIDDK
	S. Neuenschwander	Guest Worker	DB/NIDDK
	A. Koval	Visiting Scientist	DB/NIDDK

COOPERATING UNITS (if any)

J. Fontana (Univ. Maryland, Baltimore, MD)
V. Kavsan Kiev, Ukraine

LAB/BRANCH

Diabetes Branch

SECTION

Section of Molecular and Cellular Physiology

INSTITUTE AND LOCATION

National Institute of Diabetes, Digestive and Kidney Diseases, NIH 10/8D48

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5	5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Summary The insulin-like growth factors play important roles in normal growth and development. It is widely expressed in a tissue-and developmental-specific fashion, and has different functions in the various tissues and organ systems. The gene encoding IGF-I is therfore, complex with multiple controls at the transcription, translation and post-translational levels. There are two basic promoters with unique features and the alterntive usage of each promoter results in differential usage of leader exons with different leader peptides being expressed. In addition, alternative splicing of 3′ exons results in different E peptides who may undergo different post-translational (glycosylation) modifications. These differences may play a role in the differential expression and function of IGF-I in normal physiology and disease states. The IGFs are also important factors in autocrine/paracrine-induced tumor growth. Breast cancer growth is enhanced by IGFs. This effect as well as the inhibition of growth by retinoic acid, is associated with modification of the relerse of IGF binding proteins (IGFBPs). Some IGFBPs may inhibit growth, whereas, other may enhance. Thus, the IGFBPs play an important modulatory role in cancer growth.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47019 17 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Morphologic studies of ligand binding to cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I. Phillip Gorden Director NIDDK

COOPERATING UNITS (if any)

Institute of Histology and Embryology, University of Geneva School of Medicine, Geneva, Switzerland. (J.L. Carpentier, L. Orci) - Foreign

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This work represents a 17-year collaboration between the Diabetes Branch and the Institute of Histology and Embryology at the University of Geneva. The initial observations demonstrated that polypeptide hormones are taken up by the cell through a process of receptor-mediated endocytosis similar to other biologically important ligands that bind to cell surface receptors. Several lines of work have been followed in the present project. (a) We have studied the epidermal growth factor receptor, and the effect of streptozotocin diabetes in the rat on receptor-mediated endocytosis and receptor biosynthesis; (b) We have further studied the entire process of endocytosis in the diabetic rat, including both receptor-mediated and fluid-phase endocytosis; and (c) We have studied the localization of newly synthesized insulin receptors under conditions of site specific mutagenesis of glycosylation sites.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47022-14 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin Receptors in Syndromes of Extreme Insulin Resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I. S.I. Taylor Chief, DB,NIDDK

Others: D. Accili Vis. Sci., DB, NIDDK
M.L. Sierra Biologist, DB, NIDDK
M.A. Lesniak Chemist, DB, NIDDK
Y. Imai Spec. Vol., DB, NIDDK
M. Quon Clin. Assoc., DB, NIDDK
P. Gorden Director, NIDDK
E. Koller SSF, DB, NIDDK
E. Wertheimer Vis. Fel., DB, NIDDK
J. Hone Clin. Assoc., DB, NIDDK

COOPERATING UNITS (if any)

T. Kadowaki, University of Tokyo, Japan; H.Kadowaki, Tokyo, Japan; F.Barbetti, Rome, Italy; J. Roth, Johns Hopkins Medical School; M. Muggeo, Verona, Italy; E. Van Obberghen, INSERM, Nice, France; A. Cama, Chieti University, Italy.

LAB/BRANCH

Diabetes Branch

SECTION

Receptors and Hormone Action

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
10.0	8.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Insulin resistance contributes to the pathogenesis of several disease states including obesity and noninsulin-dependent diabetes mellitus (NIDDM). We have investigated the insulin receptor gene in patients with genetic forms of insulin resistance to gain insight into biochemical defects that give rise to disease.

Five classes of mutations have been identified:

1. Impaired receptor biosynthesis, due to either decreased levels of insulin receptor mRNA and/or premature chain termination mutations. In fact, we have recently identified a patient who is homozygous for a total deletion of the insulin receptor gene.
2. Impaired transport of receptors to the plasma membrane, due to missense mutations in the extracellular domain of the receptor.
3. Decreased affinity of insulin binding.
4. Decreased activity of the insulin receptor tyrosine kinase. Unlike most mutations in the extracellular domain, most mutations in the tyrosine kinase domain exert a dominant negative effect to cause insulin resistance. At least two molecular mechanisms may account for this dominant negative effect. First, by dimerizing with normal receptors, mutant receptors may inhibit tyrosine kinase activity of the oligomer. Second, by binding substrates, mutant receptors may inhibit phosphorylation of intracellular proteins by normal receptors.
5. Accelerated receptor degradation. Several mutations cause multiple defects in receptor function. For example, the Val-382 mutation in the extracellular domain impairs transport of receptors to the plasma membrane, but also inhibits the ability of insulin to activate receptor tyrosine kinase. The Glu-1135 mutation in the catalytic loop of the tyrosine kinase domain not only inactivates the tyrosine kinase, but also impairs post-translational processing and transport to the plasma membrane.

Work is underway to develop transgenic mice with insulin resistance due to mutations in the insulin receptor gene by using homologous recombination to inactivate the insulin receptor gene in embryonic stem cells. We have recently succeeded in obtaining chimeric mice.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47024-14 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biosynthetic labeling of the insulin receptor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I.	E. Collier	Medical Officer	DB, NIDDK
Others:	H. Caro	Visiting Fellow	DB, NIDDK
	P. Gorden	Director	NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

One of the post translational modifications, N-linked glycosylation, of the insulin receptor has been studied in cultured cells. Using an experimental approach based on mutagenesis of the insulin receptor Complimentary DNS (cDNA) at specific sites of potential modification, the mutated cDNA molecules are stably transfeced into cultured cells, and then the structure and function of these mutant receptors can be studied. Processing of these mutants was investigated by biosynthetic labeling. Cells unable to glycosylate their receptors in the first four potential glycosylation sites are abnormally processed. These receptors do not appear on the cell surface and remain in proreceptor form in the endoplasmic reticulum. Mutants for each of these sites, individually and in combination, also are abnormally processed. However, the severity of the defect is dependent on the site of the mutation and the number of sites mutated. The first or the second site can be mutated with very little effect on the concentration of receptor processed. However, combination of mutation in both of these sites has a dramatic effect on the processing of the receptor, whereas, the the combination of mutations in the third and fourth sites has less of an effect on the processing of the receptor.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 DK 47026- 09 DB

PERIOD COVERED
October 1, 1992 to Septembe 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Tyrosine-specific protein kinase activity associated with the insulin receptor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I.	Simeon Taylor	Chief	DB, NIDDK
Others:	Domenico Accili	Vis. Scientist	DB, NIDDK
	Sonia Najjar	IRTA	DB, NIDDK
	Neubert Phillipe	Biological Aide	DB, NIDDK

COOPERATING UNITS (if any)
University of Catanzaro, Italy (Nicola Perrotti)
Ron Margolis, DDEM, NIDDK

LAB/BRANCH
Diabetes Branch

SECTION
Receptors and Hormone Action

INSTITUTE AND LOCATION
NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In the first step in insulin action, insulin binds to its receptor on the surface of the target cell. The insulin receptor is a transmembrane protein that possesses tyrosine-specific protein kinase activity. When insulin binds to the extracellular domain of the receptor, this activates the receptor tyrosine kinase activity. A growing body of evidence suggests that the activation of the receptor's tyrosine kinase is a necessary step in initiating the biological actions of insulin. Accordingly, we have embarked upon a search for intracellular proteins that are substrates for phosphorylation by the receptor-associated tyrosine kinase. We have identified one such substrate in rat liver plasma membranes: a glycoprotein with an apparent molecular weight of 120,000 (pp 120). In addition to being a substrate for the insulin receptor, ppl20 can be phosphorylated by the receptors for epidermal growth factor and insulin-like growth factor I. pp 120 is present in liver from several species, but has not been identified in other tissues. The glycoprotein (ppl20) was immunoaffinity-purified using monoclonal antibody HA4. Based on partial amino acid sequence data, ppl20 has been tentatively identified as ectoATPase - an enzyme associated with hepatocyte plasma membranes.

We have cloned the rat gene encoding ppl20/ectoATPase. The gene contains 9 exons, and spans approximately 15 kilobase pairs of DNA. Exon 7 undergoes variable splicing. The transcript lacking exon 7 encodes an isoform in which the cytosolic domain is truncated to only 10 amino acids. The truncation deletes all three putative phosphorylation sites. When the cDNA encoding the full length isoform is expressed by transfection in 3T3 cells, it is capable of being phosphorylated by the insulin receptor tyrosine kinase. In addition, we have cloned the cDNA encoding rat pp 120/ecto-ATPase and have expressed the cDNA by transfection into cultured cells. This will enable studies of the effects of phosphorylation upon the function of the protein.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47027 - 08 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Use of SMS 201 - 995 in hormone secreting tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I. R. Eastman Director DDEMD,NIDDK

Others: C.M. Hendricks Bio. Lab Tech. DB,NIDDK

P. Gorden Director NIDDK

P. Roach Clinical Assoc. DB,NIDDK

COOPERATING UNITS (if any)

B. Weintraub Chief, MCNEB, NIDDK

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This is a project to study the long-term effects of somatostatin analog treatment on acromegaly. Patients are treated unblinded with somatostatin analog as long as it is medically necessary. Treatment and prevention of gallbladder sludge and gallstones, common side effects of somatostatin analogue treatment, is being done with ursodeoxycholate, a bile salt analog that dissolves gallstones. Patients taking somatostatin analogue are treated with ursodeoxycholate if they have gallstones or sludge, and patients without these complications are randomized for placebo controlled treatment with ursodeoxycholate to attempt to prevent sludge formation. Five patients have been randomized to masked treatment with ursodeoxycholate, and seven patients have been treated unmasked for sludge or stones. To date pre-existing gallstones or sludge have dissolved in 3 of 4 patients with gallstones and 1 of 3 patients with sludge. We continue to enroll patients in the randomized prospective trial.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47028 - 04 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transcriptional Regulation of the Insulin Receptor Gene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I.	Simeon Taylor	Chief	DB, NIDDK
Others:	Gillian Walker	Spec. Vol.	DB, NIDDK
	Hui Chen	Spec. Vol.	DB, NIDDK
	Domenico Accili	Vis. Assoc.	DB, NIDDK
	Michael Quon	Clin. Assoc.	DB, NIDDK

COOPERATING UNITS (if any)

Catherine McKeon, DDEM, NIDDK

LAB/BRANCH

Diabetes Branch

SECTION

Receptor and Hormone Action Section

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Regulation of the number of insulin receptors on the cell surface plays a critical role in determining insulin sensitivity. In order to study the mechanism of transcriptional regulation of the insulin receptor gene, we have cloned the 5' end of the human and mouse insulin receptor gene. We have begun to characterize the proximal promoter and find it has many features of a "housekeeping gene". In addition, we have localized a weak enhancer upstream of the promoter that is conserved in both the human and mouse promoters. This enhancer sequence binds nuclear proteins from many different cell lines. The proximal promoter is probably responsible for the low level expression of the insulin receptor gene which occurs in most cell types. Recently we have identified a region of the first intron which may be involved in tissue specific regulation. This region seems to be responsible for the 10-fold induction of the insulin receptor gene during adipocyte differentiation in 3T3-L1 cells in vitro. We inquired whether this sequence in the first intron had the same function. To address this question, we constructed transgenic mice in which a reporter gene (chloramphenicol acetyl transferase) is driven by the insulin receptor promoter plus the putative regulatory element in intron 1. However, the CAT reporter gene was expressed primarily in brain. Little if any CAT activity was detected in adipose tissue. Thus, 3T3-L1 cells in vitro appeared not to be a perfect model for adipose tissue in vivo. Accordingly, we have developed a system for transfecting primary cultures of rat adipocytes. This system can be used to map cis-acting elements in the promoter using expression vectors with a luciferase reporter gene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47029 - 02 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical modeling of glucose metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I.	M. Quon	Clinical Associate	DB,NIDDK
Others:	R. Eastman	Director	DDEMD,NIDDK
	S.I. Taylor	Chief	DB,NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Diabetes Branch

SECTION

Receptors & Hormone Action

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Insulin resistance occurs in a variety of pathological states including obesity, non-insulin dependent diabetes mellitus (NIDDM) and hypertension. Decreased glucose effectiveness has been reported in NIDDM. Since insulin resistance and impaired glucose effectiveness may play a role in the pathogenesis of NIDDM, quantitative assessment of these properties is of interest. Recently, a minimal model approach involving mathematical modeling and computer simulation has been used to estimate both insulin sensitivity and glucose effectiveness from the results of a single frequently samples intravenous glucose tolerance test (FSIVGTT). The equations of the minimal model describe changes in plasma glucose concentration as functions of insulin and glucose concentrations. A computer program identifies model parameters that generate a best fit to insulin and glucose data obtained during the FSIVGTT. Thus, the minimal model is able to estimate the relative contributions of insulin and glucose to glucose tolerance. We have used the minimal model to generate specific predictions of both insulin-dependent and insulin-independent glucose metabolism under a variety of conditions. We performed experiments in subjects with insulin dependent diabetes mellitus and in normal subjects to test these predictions. By comparing model predictions with experimental results, we were able to demonstrate that the minimal model underestimates the contributions of insulin and overestimates the contribution of glucose to glucose metabolism. Studies are presently underway to confirm these results and to understand the origin of the discordance between minimal model predictions and experimental results. The data that we collect may allow us to formulate a more accurate and useful mathematical model.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47030 - 02 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin-receptor related receptor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I.	S. Taylor	Chief,DB/NIDDK
Others:	D. Accili	Visiting Scientist,NIDDK
	H.Y. Jui	Special Volunteer,NIDDK
	Y. Suzuki	Special Volunteer,NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Diabetes Branch

SECTION

Receptors & Hormone Action

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The insulin receptor is encoded by a single copy gene located on chromosome 19 in the human. In addition, at least two other highly homologous genes have been identified. One gene encodes the receptor for insulin like growth factors (IGF)-I and -II. The third gene in the family encodes another receptor tyrosine kinase. The ligand for this third receptor has not been identified; however, preliminary evidence suggests that this insulin-receptor related receptor (IRR) does not bind insulin, IGF-I, or IGF-II. The goals of this project are two-fold: (1) to identify the ligand for this receptor, and (2) to elucidate the physiological role of the IRR. Toward this end, we have cloned human IRR cDNA. This work revealed that the cDNA undergoes differential splicing; some of the transcripts (<10%) use an alternate splice acceptor site that is located 24 nucleotides upstream from the predicted 5'-end of exon 14. Use of this alternative splice acceptor site preserves the reading frame in human IRR cDNA, this would not be the case in the mouse gene since the site is located 23 nucleotides upstream from exon 14. In addition, multiple alternative splicing patterns were identified in exons 1 and 2 of the murine gene. None of these preserve the reading frame. The function of these alternative splicing patterns has not been elucidated. By expressing IRR cDNA in cultured cells, we hope to develop a bioassay for the IRR ligand. In addition, we have cloned the murine IRR gene. A fragment of the cloned gene has been used to construct a targeting vector that is being used to inactivate the IRR gene by homologous recombination in embryonic stem cells. Once this is successful, we will attempt to construct transgenic mice with mutations in the IRR gene. These transgenic mice will be studied in an effort to determine the phenotypic effect of mutations in the IRR gene. This has the potential to provide clues into the physiological roles of the IRR and its ligand.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47031-02 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I.	M. Reitman	Senior Staff Fellow	DB, NIDDK
Others:	Lynne Abruzzo	Clinical Associate	DB, NIDDK
	Mark Mason	IRTA	DB, NIDDK
	Katherine Poehling	Special Volunteer	DB, NIDDK

COOPERATING UNITS (if any)

Gary Felsenfeld, LMB, NIDDK; Heiner Westphal and Eric Lee, LMG, NICHD; Joseph Grasso, University of Connecticut Health Center

LAB/BRANCH

Diabetes Branch

SECTION

Molecuar Biology and Gene Regulation

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our goal is to understand two aspects of gene regulation: 1) the mechanisms that operate to 'open' chromatin and 2) those that dictate how a particular gene within an open multi-gene cluster is chosen for expression. The model evolving from studies of the human β-globin cluster is that control elements upstream of the genes provide a locus activation function. These upstream elements also increase the expression of the nearest available genes, with availability determined by the promoter. Human diseases with defects in each of these processes are known (eg. β-thalassemia (Hispanic form) and hereditary persistence of fetal hemoglobin). Knowledge of these topics is part of the background needed for a rational approach to gene therapy.

We previously demonstrated that the chicken β^A-globin gene and its 3' enhancer contain information sufficient to guarantee copy-number dependent expression in transgenic mice, independent of the site of transgene integration (this property defines a locus control region, LCR). To study the way in which an enhancer/locus control region activates chromatin, we examined transgenic mice carrying various combinations of the chicken β^A-globin gene coding region, promoter and 3' enhancer/LCR. The results support a 'mutual interaction' model for the mechanism of chromatin opening by LCRs in which an enhancer/LCR and a promoter must cooperate to generate open chromatin.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 48001-01 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin-Cell Interaction

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

PI:	Ian A. Simpson	Visiting Scientist	DB/NIDDK
Others:	Samuel W. Cushman	Chief, EDMNS	DB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Diabetes Branch

SECTION

Experimental Diabetes, Metabolism, and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Inactive.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 DK 48002-02 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Insulin's Regulation of Glucose Transport

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

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Others: I. A. Simpson, Visiting Scientist, DB/NIDDK; F. Maher, Visiting Fellow, DB/NIDDK; M. Millo, Special Volunteer, DB/NIDDK; K. I. Timmers, Special Volunteer, DB/NIDDK; C. M. Wilson, IRTA, DB/NIDDK; M. Omatsu-Kanbe, Special Volunteer, DB/NIDDK; T. Ploug, Special Volunteer, DB/NIDDK; J. T.Brozinick, IRTA, DB/NIDDK; K. Sango, Visiting Fellow, DB/NIDDK; D. R. Yver, Chemist, DB/NIDDK; P. A. Ortiz, Special Volunteer, DB/NIDDK; S. J. Vannucci, IPA, DB/NIDDK; M. J. Zarnowski, Biologist, DB/NIDDK; M. J. Quon, Clinical Associate, DB/NIDDK

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Diabetes Branch

SECTION

Experimental Diabetes, Metabolism, and Nutrition Section

INSTITUTE AND LOCATION

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TOTAL STAFF YEARS	PROFESSIONAL	OTHER
11.1	11.1	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

The subcellular trafficking of tracer-tagged GLUT4 between the plasma membranes and low-density microsomes of rat adipose cells has been studied. Cell-surface GLUT4 have been initially tracer-tagged in the insulin-stimulated state with a [3H]-bis-mannose. The initial experiments show that insulin does not alter the half-time for GLUT4 endocytosis but instead increases the rate of exocytosis. Additional data suggest that the cells' entire complement of GLUT4 is involved in the recycling process. Finally, detailed time-course data suggest that there may be plasma membrane intermediate states in the GLUT4 trafficking pathways. Peptides from the alpha 1 domain of the major histocompatibility complex class I antigen (MHC class I) enhance cellular glucose uptake above that of maximal insulin stimulation, prolong the effect of insulin, and inhibit insulin receptor internalization in rat adipose cells. Based on the new data here, we now propose that MHC class I molecules may be involved in regulation of the internalization process of cell surface integral membrane proteins such as the glucose transporter, IGF-II receptor, and insulin receptor. We have also found that EGF in combination with certain MHC class I-derived peptides is insulinomimetic and that this effect is independent of insulin receptor activity. In order to obtain reliable kinetic constants for the two glucose transporter isoforms found in insulin responsive tissues, each transporter was expressed in Xenopus oocytes by the injection of mRNA encoding rat GLUT1 or GLUT4. The 3-O-methylglucose kinetic data indicate that, at low substrate concentrations, the catalytic efficiency of GLUT4 is significantly greater than GLUT1. Extrapolation to mammalian systems suggests that GLUT4 is responsible for virtually all of the hexose uptake in insulin responsive targets, particularly in the presence of hormones. In studies of the effects of K^+ depletion, we observed an inhibition of GLUT4 internalization which is entirely analogous to the effects on IGF-II/Man-6-P receptor cycling strongly suggesting the involvement of a coated pit mechanism in the recycling of GLUT4 transporters. An inactive conformation of GLUT4 has also been detected in plasma membranes from insulin-stimulated cells which is enhanced by K^+ depletion without a corresponding increase in transport activity suggesting a limit in the adipose cells' capacity to promote active GLUT4 transporters.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 DK 48003-02 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Alterations in Insulin's Action in Insulin-Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI: Samuel W. Cushman Chief, EDMNS DB/NIDDK

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LAB/BRANCH

Diabetes Branch

SECTION

Experimental Diabetes, Metabolism, and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS	PROFESSIONAL	OTHER
0.0	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Inactive.

PHS 6040 (Rev 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 48005-02 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Counterregulation of Insulin's Action by Catecholamines

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Others:	Susan J. Vannucci, Ph.D.	IPA	EDMNS/DB/NIDDK
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LAB/BRANCH

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TOTAL STAFF YEARS	PROFESSIONAL	OTHER
0.0	0.0	0.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Insulin-stimulated glucose transport activity in rat adipocytes is inhibited by isoproterenol and enhanced by adenosine. Both of these effects occur without corresponding changes in the subcellular distribution of the GLUT4 glucose transporter isoform. In this report, we have utilized the impermeant, exofacial bis-mannose glucose transporter-specific photolabel, 2-N-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA), to examine the cell surface accessibility of GLUT4 glucose transporters under these conditions. Compared to cells treated with insulin alone, adenosine in the presence of insulin increases the accessibility of GLUT4 to the extracellular photolabel by ≈25% consistent with its enhancement of insulin-stimulated glucose transport activity; the plasma membrane concentration of GLUT4 as assessed by Western blotting is unchanged. Conversely, isoproterenol, in the absence of adenosine, promotes a time-dependent ($t_{½}$≈2 min) decrease in the accessibility of insulin-stimulated cell surface GLUT4 by >50%, which directly correlates with the observed inhibition of transport activity; the plasma membrane concentration of GLUT4 decreases by 0-15%. Photolabeling the corresponding plasma membranes revealed that these alterations in the ability of the photolabel to bind to GLUT4 are transient as the levels of both photolabel incorporation and plasma membrane glucose transport activity were consistent with the observed GLUT4 concentration. These data suggest that insulin-stimulated GLUT4 glucose transporters can exist in two distinct states within the adipocyte plasma membrane, one functional and accessible to extracellular substrate and one non-functional and unable to bind extracellular substrate. These effects are only observed in the intact adipocyte and are not retained in isolated plasma membranes isolated from these cells when analyzed for the ability to transport glucose or bind photolabel.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 48007-01 DB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Glucose Transport in Mammalian Brain

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

P.I. Ian A. Simpson, Ph.D.	Visiting Scientist	EDMNS/DB/NIDDK
Frances Maher, Ph.D.	Visiting Fellow	EDMNS/DB/NIDDK
Susan J. Vannucci, Ph.D., I.P.A.	(Intergovernmental Personnel Agreement	EDMNS/DB/NIDDK
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Diabetes Branch

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3.7	2.4	1.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Three distinct glucose transporter isoforms have been detected in mammalian brain. A 55kDa GLUT1 isoform is the primary transporter in the blood-brain barrier. A lower molecular weight GLUT1 (45kDa) has been observed in vessel-free cortex and is presumably of neuronal and or glial origin. We have previously shown that GLUT3 is present in primary neuronal cultures and more recently in situ hybridization studies have confirmed its presence in neurons in intact tissue. Most recently, we, in collaboration with Dr. Peter Davies, have detected GLUT5 immunohistochemically in human microglia and also in human monocytes following their differentiation into tissue macrophages.

One of the major objectives of our studies has been to investigate what effects diabetes has on the region-specific expression of these glucose transporters in brain. We have employed two animal models of diabetes: 1) the streptozotocin-treated rat and, 2) in conjunction with Dr. Robert Sherwin at Yale University, the BB-Wistar rat. In the first model we found a diabetes-induced increase (20%) in the level of GLUT3 protein in the neurohypophysis which was detected by day 3 following administration of streptozotocin and persisted through four weeks. Conversely, GLUT1 was decreased by 20% by day 3 and was 53% of the control level at 4 weeks. This represents the first demonstration of stress-related regulation of GLUT3 expression.

In the BB-Wistar rats we studied the effects of diabetes and episodic hypoglycemia on region-specific glucose transporter expression. In the diabetic animals the levels of GLUT1 were increased 18-63% in temporal cortex, frontal cortex, hippocampus and brain stem and decreased in cerebellum (10%) and pineal (40%). GLUT3 was found to be increased (18-38%) in hippocampus, brain stem and neurohypophysis. However, recurrent hypoglycemia had no effect on the expression of either GLUT1 of GLUT3 in either control or diabetic animals. Thus, in contrast to peripheral tissues where there is a down-regulation of transporters associated with diabetes, in brain an up-regulation is observed.

ANNUAL REPORT OF THE CLINICAL HEMATOLOGY BRANCH

National Institute of Diabetes and Digestive and Kidney Diseases

Study of Immunology of Blood Cell Deficiencies

Objectives:

To study the immunochemistry of hematologic disorders involving sensitization to autoantigens, alloantigens, and drugs, and the effects of the immune reactions on cellular physiology, biochemistry, and *in vivo* cellular kinetics.

Methods employed:

Gel filtration of platelets; platelet activation by thrombin and other agonists; flow cytometric measurement of platelet- and red cell-associated Igs; platelet serotonin and ATP secretion studies; monoclonal antibody antigen capture assays; fibrinogen binding by platelets; lectin and monoclonal affinity chromatography for platelet glycoprotein purification; anion exchange chromatography; SDS-PAGE and native gel electrophoresis; protein immunoblotting; PVDF blotting of proteins for sequencing; purification of antibodies by adsorption/elution; immunoprecipitation; complement fixation; isoelectric focusing; electron microscopy; cryoprecipitation; thin-layer and high-pressure chromatography for identification of platelet microparticle-associated phospholipids; molecular sizing chromatography; elutriation; PCR amplification of modified platelet GP cDNA and expression of GPs for studies of autoAb epitope specificities.

Immunologically-mediated cell destruction is the principal cause of thrombocytopenia and a major cause of hemolytic anemia. Autoimmunity underlies most syndromes, but sensitization to foreign antigens is the basis for many, e.g., drug-dependent cytopenias, posttransfusion purpura (PTP), and thrombocytopenia of certain infections.

I. Autoimmune syndromes

Idiopathic Thrombocytopenic Purpura (ITP), the most common acquired hemorrhagic disorder, affects approx. 250,000 individuals in the U.S. Sensitizing antigens are not known, diagnostic serology is not established, and therapy remains empirical. Recently, platelet glycoprotein (GP) targets of autoantibodies (autoAbs) have been identified by antigen-capture techniques. Serologic studies of others have focused on IgG Abs against GP IIb-IIIa. Abs were detected in approx 50% of ITP sera, but most appeared to be nonpathogenic because they were directed against cytosolic (internal) epitopes.

Findings:

We evaluated sera from 47 patients with chronic ITP for IgA, IgM, and IgG Abs against four major platelet membrane GP complexes,

differentiated cytosolic and exosolic epitopes, determined Ab titers, and correlated results with clinical status. 85% of sera reacted with one or more GP; IgA reactions were as common as IgG; IgM agglutinins were in 15% of sera. Although many Abs were directed against internal GP epitopes, when all classes against all GPs were considered, 80% of sera had at least one Ab against an external GP epitope. Titers were often high, and affinities, compared to those of alloantibodies, low. Neither Ab class nor GP specificty of Ab clearly correlated with refractoriness to therapy. Contrary to a previous report, we found Abs against internal GP epitopes prevalent in patients recovered from any of several disorders involving platelet destruction. Furthermore these Abs are not bound by intact platelets and likely nonpathogenic. In preliminary studies, Abs directly eluted from platelets of patients with ITP closely matched specificities and classes of serum Abs against exosolic Ags.

Planned Work:

1. To use site-deleted recombinant GP Ib to further characterize four novel exosolic epitopes of GP Ib reactive with ITP sera.
2. To directly measure platelet-associated Igs of all classes, a method which may prove easier for routine evaluation of ITP patients.
3. To evaluate cause of increases (up to 20-fold) of platelet-associated proteins in disorders of platelet destruction by quantitating protein-rich platelet membranous material that adsorbs to intact platelets (see PTP below) and further utilize a dog model we developed to duplicate the phenomenon.
3. To apply principles of ITP serology to platelet destructive disorders, including HIV-associated thrombocytopenia and refractoriness to platelet transfusion.

II. Drug-induced immune cytopenias

Immunologically mediated adverse reactions to drugs account for 2 to 4% of hospital admissions. Syndromes in which blood cells are destroyed by drug-dependent Abs (ddAbs) provide rare but excellent opportunities to study mechanisms of cytotoxicity. There is controversy concerning the nature of the immunogenic stimulus in these syndromes and of bimolecular low affinity reactions leading to ternary high-affinity antibody-drug-cell complexes. Recently, major membrane GPs have been found to participate in platelet reactions and we have found red cell Band 3 to be involved in 3 cases of drug hemolytic anemia. It has been suggested that labile associations between drug molecules and GPs induce immunogenic GP neoantigens that are targets of resultant antibodies. We find from stoichiometric studies that drug is the primary antigen and proteins participate through conformational induction.

Findings:

We utilized highly purified concentrated antibodies from patients with drug purpura and drug hemolytic anemia in equilibrium

dialysis against radiolabeled drug, with and without cell membranes. The known low affinity between membranes and drug ($K_a<10^3$ M^{-1}) and between antibody and drug ($<10^5 M^{-1}$) increased progressively when Ab and membranes were exposed to increasing concentrations of drug. Apparent affinity between any two of 3 reactants in equilibrium antibody-drug-membrane complexes varied from 10^5 to 10^6 M^{-1} at 5×10^{-8} M drug and from 10^7 to 2×10^8 at 10^{-5} M drug. At lower drug concentrations, the ratio of drug to antibody was 1:1 and did not exceed 2:1 at optimal drug concentrations for antibody binding. These findings are consonant with our previous demonstration that affinity of ddAb binding by the $F(ab')_2$ domain is 1-2 orders of magnitude greater than binding by Fab monomer (JCI 79:912, 1987). Moreover, by competitive studies, we found that antibodies with different drug specificities can bind at the same GP site. The primary epitope of drug antibodies appears to be drug, the concentration of which determines the valence (affinity) of antibody binding to cell sites. Antibody and combining site flexibility leading to induced conformational complementarity may account for the high affinity ternary complex. The immunogen could be a classical covalent complex of reactive drug metabolite with any protein.

Planned Work:

1. To identify site(s) on Band 3 that bind ddAbs and determine whether the sites are drug-specific.
2. To determine ddAb affinity for drugs by fluorescence quenching and other measures of conformational transition due to ligand binding.
3. To substitute purified GPs for cell membranes in stoichiometric and kinetic studies.
4. To further study competition by different ddAbs for GP binding sites.

III. Posttransfusion purpura (PTP)

This syndrome, described by Shulman, et al(JCI 40:1597,1961), is characterized by severe thrombocytopenia developing 5 to 8 days after transfusion of platelets or platelet material into a recipient lacking a transfused platelet alloantigen. Antigen (Ag)-Ab complexes were thought to be responsible, but others suggested autoAbs or crossreactive alloAbs.

Findings:

We recently found: Ultracentrifuged plasma contains 0.5 to 1.5% of the alloantigen (e.g. Pl^{A1}) content of intact platelets in an equivalent volume of platelet-rich plasma (PRP). Nonsedimentable Pl^{A1} Ag adsorbs onto Pl^{A1}-negative platelets equally well in the presence or absence of anti-Pl^{A1}, the former representing the clinical situation. Pl^{A1} Ag was demonstrated in acute-phase plasma of patients with PTP whose own platelets after recovery lacked that Ag. To do this, a method of extracting Ag from large volumes of plasma was devised employing

cryoprecipitation and lectin chromatography. Amounts of Ag in 5 patients varied from 0.03 to 0.1% of that in an equal volume of normal Pl^{A1}-positive PRP, which provides much more than the 300 Ag-Ab complexes per platelet estimated as necessary to cause platelet destruction. Infusion of either affinity-purified anti-Pl^{A1} or Pl^{A1}Ag into guinea pigs did not affect platelet counts, but infusion of Ag followed by excess Ab or preformed Ag-Ab complexes caused decreases in platelets that were dependent on Ag dose. By exclusion chromatography, Ag was in the void volume and also in a soluble proteins peak. Electron micrographs, utilizing gold immunolabelling techniques, show apparent particulate and soluble Ag adsorbed on platelet membranes.

Planned Work:

1. To further characterize nonsedimentable platelet fractions physically and chemically, and determine their effects on platelet function.
2. See section I, plan 3 regarding platelet-associated proteins.

Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

Objectives:

To further define the nature of blood coagulation by studying reactions and interactions of coagulation factors *in vitro* and *in vivo*, and the physiologic and biochemical platelet responses to stimuli causing exocytosis and aggregation.

Methods Employed: Methods of protein purification and characterization; kinetic analysis of enzyme reactions; procedures for quantitative measurement of various clotting factors; assessment of metabolic pathways of blood cells with radioactive substrates, particularly arachidonate metabolism and tyrosine phosphorylation; tissue culture; pharmacologic and physiologic studies of cellular secretion, and platelet aggregation; SDS-PAGE; amino acid composition of purified proteins; flow cytometry; protein immunoblotting, EIA, RIA, and other quantitative and qualitative methods of identifying specific epitopes with antibodies; HPLC; affinity columns; immunoprecipitation; isoelectric focusing.

I. Vinculin is a Major Platelet Protein that Undergoes Calcium-dependent Tyrosine Phosphorylation

Findings:

Platelet activation by strong agonists like thrombin is associated with tyrosine phosphorylation of a 130 kD protein and, to a lesser extent, of 80 and 60 kD proteins. This phosphorylation appears to be due to activation of a cytoplasmic tyrosine kinase by elevated cytosolic calcium (Ca_i) as well as to inhibition of tyrosine phosphatase activity by depletion of calcium stores (Vostal, Jackson and Shulman, JBC 266:16911-16916, 1991). Dephosphorylation of these proteins occurs apparently as a result of reactivation of tyrosine phosphatase when calcium stores are repleted through microsomal calcium ATPase. Thus the levels of Ca_i and stored calcium appear to antagonistically control tyrosine phosphorylation of specific platelet proteins, the most prominent of which has a MW of 130 kD, and is not separable from other platelet membrane proteins, notably GP IIb, by SDS-PAGE. We found that a major protein in the 130 kD range reacted with both monoclonal anti-phosphotyrosine and anti-vinculin. We have further found that the protein could be immunoprecipitated or purified by solid phase separation using these Abs. Vinculin and the 130 kD tyrosine-phosphorylated protein are identical in physical characteristics of molecular weight, solubility in nonionic detergent, lack of N-linked glycosylation, and isoelectric point. These, and our previous observation that the 130 kD protein is tyrosine-phosphorylated in a calcium dependent manner during

platelet activation suggest that vinculin is an important protein controlling platelet secretory responses.

Planned work:

The above studies were begun, in part to determine whether anti-platelet Abs induce biochemical responses that may be used as a sensitive measure of immune platelet injury. Prototypic alloimmune and autoimmune reactions will be tested for their effects on tyrosine phosphorylation.

References:

1. Reid DM, Jones CE, Luo C-Y, Shulman NR: Immunoglobulins from Normal Sera Bind Platelet Vinculin and Talin and Their Proteolytic Fragments. Blood 81:745-751, 1993.

2. Shulman NR, Reid DM: Platelet Immunology. In:Colman R, Hirsh J, Marder VJ, Salzman EW,eds. Hemostasis and Thrombosis. Basic Principles and Practice 3rd edition, JB Lippincott, NY, in press, 1993.

3. He RY, Reid DM, Jones CE, Shulman NR: Spectrum of Ig Classes, Specificities, and Titers of Serum Anti-glycoproteins in Chronic Idiopathic Thrombocytopenic Purpura (ITP). Submitted to Blood, 1993

4. Shulman NR, Reid DM: Mechanisms of Drug-induced Immunologically Mediated Cytopenias. Trans Med Rev, in press, 1993.

5. Vostal JG, Shulman NR: Vinculin is a Major Platelet Protein that Undergoes Calcium-dependent Tyrosine Phosphorylation. Biochem J, in press, 1993.

DEPARTMENT OF HEALTH AND HUMAN SERVICES–PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 51006-35 CHB

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT(80 characters or less. Title must fit on one line between the borders.)

Study of Immunology of Blood Cell Deficiencies

PRINCIPAL INVESTIGATOR(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.65	2.9	0.75

CHECK APPROPRIATE BOX(ES)

[x] (a) Human Subjects [x] (b) Human Tissues [ ] (c) Neither

[ ] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK(Use standard unreduced type. Do not exceed the space provided.)

Immune-mediated thrombocytopenias are the leading cause of hemorrhagic diathesis with a prevalence >0.1%. Autoimmune idiopathic thrombocytopenic purpura (ITP) is the most common, but sensitization to alloantigens and drugs cause some of the severest thrombocytopenias. Mechanisms underlying these disorders are obscure.

We found ITP antibodies (Abs) to be directed against platelet membrane glycoproteins(GPs) IIb/IIIa and Ib/IX equally and less often against GPs Ia/IIa and IV; IgG and IgA classes were present equally and IgM rarely. Abs against exosolic domains of GPs were unique to ITP. Abs to cytosolic domains were a nonspecific secondary phenomenon of platelet destruction. Five GP peptides of 12 to 23 amino acids, selected for potential immunogenicity, defined epitopes recognized by different ITP sera. Further epitope analysis is underway using recombinant GP fragments.

Posttransfusion purpura (PTP) was found to be caused by adsorption of platelet membrane material from transfused blood to recipients' platelets which are then destroyed by alloAbs that develop against the transfused material. The nonsedimentable material was both particulate (<0.1 μm), associated with a group of platelet- and plasma-derived proteins, and soluble, in association with phosholipids. Human platelet membrane-derived material coated guinea pig platelets in vivo and as little as 200 molecules of antigen per cell resulted in platelet destruction by human alloAbs. By electron microscopy using gold-labeled Ab probes, human material adsorbed by guinea pig platelets was distributed like integral GPs on human platelets.

In drug hemolytic anemia, 2 cases caused by stibophen and one by tolmetin had drug-dependent antibodies that reacted with erythrocyte band 3. No major blood group substance was involved in the reaction. The submolecular component(s) participating in the drug-dependent reaction will be investigated utilizing fragmented and enzyme-altered band 3.

DEPARTMENT OF HEALTH AND HUMAN SERVICES-PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 51001-35 CHB

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT(80 characters or less. Title must fit on one line between the borders.)

Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

PRINCIPAL INVESTIGATOR(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Raphael Shulman	Chief	CHE:NIDDK
Others:	Charles E. Jones	Chemist	CHB:NIDDK
	Diane M. Reid	Expert	CHB:NIDDK

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.35	0.10	0.25

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[x] (a) Human Subjects [x] (b) Human Tissues [ ] (c) Neither

[ ] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK(Use standard unreduced type. Do not exceed the space provided.)

Platelets prevent hemorrhage by a secretory process controlled through an intracellular chain of bichemical events similar to that in all other secretory cells. Tyrosine kinase (TK) activity, which causes tyrosine phosphorylation (TP) of specific cellular proteins is temporally associated with secretion by platelets and other cells, as well as with cellular responses such as growth, contact inhibition, and malignant transformation. The role of TP in these processes is not known. We previously found that increased platelet cytoplasmic calcium accompanied secretion and TP of a 130 kD major cytoplasmic protein, whereas return of calcium to hemostatic levels in storage compartments promotes tyrosine dephosphorylation of this protein (JBC 226:16911-16916, 1991). We identified the 130 kD platelet protein as most likely being vinculin by its reaction on immunoblot and by affinity chromatography isolation with both antibodies. This year we firmly established that the protein is vinculin by immunoprecipitation with anti-vinculin and anti-phosphotyrosine, by isoelectric focusing to determine the pI, and by ruling out the presence of glycoprotein IIb/IIIa and CD31 which travel in immunoblots at approximately the same level as vinculin.

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

Biochemical Genetics Section

Dr. Bernstein has initiated studies to investigate the process whereby newly synthesized proteins are recognized and targeted to the endoplasmic recticulum (ER) in eukaryotic cells or to the cytoplasmic membrane in procaryotic cells. The project employs biochemical and genetic approaches in the functional analysis of the signal recognition particle (SRP) and the SRP receptor in the initial stages of protein export. Mutants of SRP have been created to elucidate the mechanism of signal sequence recognition by SRP as well as the role of the GTPase activity associated with SRP in regulating the entry of proteins into the secretory pathway. Additional studies have been initiated to understand the function of SRP and SRP receptor homologs in *E. Coli* where facile genetic analysis is possible. These studies should yield insight into the mechanisms of protein sorting into cellular organelles, a process central to all cells.

Dr. Proia and co-workers have continued their work on the G_{M2} gangliosidoses, a group of well-studied disorders that typify the more than 30 other lysosomal storage diseases. In order to create mouse models of the diseases, the group is inactivating the hexosaminidase genes in mouse embryonic stem cells by homologous recombination. Thus far they have disrupted the HEXA gene, which causes Tay-Sachs disease when mutated in humans, and have produced mice homozygous for the disrupted allele. The mice display a complete deficiency of β-hexosaminidase A, the enzyme absent in Tay-Sachs disease and may provide an suitable amimal model for the study of gene therapy in this disorder. The group is also studying the structural determinants that provide the different substrate preferences of the hexosaminidase isozymes. By creation of chimeric enzymes, the substrate specificity region of the α subunit has been shown to reside in two discontinuous portions of the polypeptide. This research should increase our understanding of a key component of the lysosomal degradative machinery, as well as, provide the framework for the development of novel therapies for the treatment of lysosomal disorders.

Molecular Genetics Section

Dr. Ackerman and colleagues have extended their studies on DNA repair and replication. They have demonstrated that two distinct types of UV-damage to DNA are efficiently repaired in both injected *Xenopus* oocytes and in a nuclear extract derived from oocytes. They have demonstrated roles for various accessory proteins involved in these reactions by adding antibodies to putative accessory proteins to repair/replication reactions and then reversing the inhibitory effects of the antibodies by adding purified proteins back to the reactions.

In addition, Dr. Ackerman and colleagues have continued to study the molecular basis of bypass replication past damaged bases on DNA substrates as well as continued to characterize how angiogenin specifically hydrolyzes on tRNA when injected into Xenopus oocytes or added to a rabbit reticulocyte lysate.

Dr. Hsieh and her colleagues.have been studying the process of DNA branch migration, a process whereby two homologous DNA duplexes exchange strands and an essential component of genetic recombination. They previously demonstrated that a single base mismatch blocks nonenzymatic branch migration. Since homologous recombination regularly involves the exchange of DNA strands between two similar but not identical duplexes, their result suggested that recombination proteins are required to facilitate branch migration through sequence heterology. In order to further characterize the branch migration step, they developed an assay to determine kinetic parameters of spontaneous branch migration. The time required for the Holliday junction to move one base pair was measured as a function of temperature and ionic conditions. Branch migration is three orders of magnitude slower in the presence of Mg^{2+} compared to Na^{+}. They attribute this effect to the way in which metals influence the conformation of the four-armed Holliday junction. Subtle differences in the conformation of the junction can greatly effect the overall rate of branch migration. Their results establish that spontaneous branch migration is too slow to account for heteroduplex formation in biological systems and underscore the importance of proteins in promoting branch migration during recombination.

Dr. Camerini-Otero and his colleagues continue their studies aimed at dissecting the biochemical steps involved in genetic recombination. Over the last few years they have focused on identifying the proteins responsible and how they work. They have chosen to concentrate their efforts on a key early step: strand exchange between homologous parental DNAs. *In vitro*, the product of this exchange is joint molecule composed of a single-strand DNA joined to the end of a duplex DNA. First they identified several eukaryotic proteins responsible for this reaction. They have established a new paradigm of this homologous pairing: that all recombinases, including the *E.coli* recA protein, can hybridize a single strand of any sequence and an intact duplex. That is, the three strand for a novel DNA triplex (which they have designated R-form DNA) in which the third strand may include both purines *and* pyrimidines. Recently, they have used thermal denaturation of chemically substituted DNAs and chemical footprinting of R-form DNA to confirm that the third strand in R-form DNA is in the major groove of the duplex.

Dr. Camerini-Otero in collaboration with Drs. Victor Zhurkin and Bob Jernigan of the NCI have proposed a new model for this recombination triplex DNA, a structure that they call R-form DNA. This model is based on conformational energy calculations. The calculations indicate that R-form is sterochemically possible for any arbitrary sequence and that the positions of all four bases in the third strand are nearly isomorphic.

Dr. Camerini-Otero and colleagues have also been able to significantly extend the extent of non-enzymatically formed DNA triplexes. In their investigations they have been able to determine that alterations in the minor grrove very often accompany the docking of the third strand into the major groove. This insight might prove very valuable in manipulating the duplex DNA so that in the absence of recombination proteins it can accept a third strand of any sequence to form a triplex. This is a long term goal of this group.

Dr. Camerini-Otero and colleagues have also been able to isolate synaptic complexes consisting of all the three strands and recA. In most experiments the single strand is an oligonucleotide directed to a target on a larger duplex DNA. These structures have been studied in detail and will continue to be the basis of additional structural investigations. The kinetics of formation of these complexes are being used as a model for the homology search process in the obligatory recombination events during meiosis. The results so far indicate that the reaction is a second order reaction and that in the presence of suffcient RecA the rate depends on the concentrations of the single strand oligonucleotide

DNA and the duplex DNA target sites. That is, for a given concentration of target, the rate of forming a specific complex is the same whether the target sequence is part of a short duplex of a few base pairs or it is part of the human genome. Since in this last case there is approximatley a 10^9-fold excess of nonspecific to specific sites, the scanning of nonspecific sites must be extremely fast. In most biological scenarios the search for homology must be very fast compared to other processes, such as chromosome movements or the uptake of exogenous DNA.

The synaptic complexes have also been used to develop a method for the selective cleavage of human DNA (RecA-Assisted Restriction Endonuclease (RARE) cleavage); this method has being applied to map and clone large fragments of DNA close to the Huntington Disease gene. In addition, RARE has been used to map across the notorious "gap" close to the telomere of the short arm of the human chromsome 4p, near the Huntington Disease gene. RARE has also has been used to measure the distance of several loci to the telomere and fragments ending in the telomere have been prepared.

In addition, Dr. Camerini-Otero and colleagues have been successful in cloning the gene for a thermostable recA homologue. This protein should be very useful for mechanistic studies and for several important applications in biotechnology. Finally,they have cloned from yeast and higher eukaryotes several sequence homologues to recA. They are attempting to establish functional homology between the proteins encoded by these genes and the *E. coli* recA protein.

Finally, Dr. Camerini-Otero and colleagues have defined a 35 amino acid stretch of the E. coli RecA protein as the single-strand DNA binding domain. This region includes the second (L2) of two disorganized loops in the X-ray crystal structure of this protein determined by other workers.

Mechanisms of Gene Regulation Section

Investigators in this section conduct basic scientific research on the mechanisms of action of the thyroid hormone and effects of a family of POU-domain genes on anteroposterior axis formation during amphibian embryogenesis.

Dr. Nikodem and colleagues continue to study how the rate of transcription of target genes is modulated by binding of thyroid hormone receptor-hormone complex to the specific DNA. The thyroid hormone receptor receptor α gene is alternatively spliced to give the α receptor and a variant which does not bind thyroid hormone. An additional α like receptor, Rev-erbAα, is transcribed from the opposite strand downstream of thyroid hormone receptor α but convergent over 256 bases with the variant and not the α receptor. The overlapping regions of these two transcripts raises the possibility that a sense-antisense hybrid might form in vivo and thus could modulate a level of the variant with concomitant increase in production of the α mRNA. No functional results could be seen in vivo from potential anti-sense pairing of Rev-erbAα with the variant, since the level of variant mRNA stays high from fetal to adult stage, while the Rev-erbAα is present only in adult tissues.

An additional means in controlling the action of thyroid hormone could be provided by regulating expression of the receptor gene. Dr. Nikodem and her colleagues cloned and sequenced the 5' flanking region of the receptor α and minimal promoter sequences required for expression of this gene were characterized. This promoter is GC rich and it

does not contain either a TATA or CAAT box. Furthermore the promoter activity is down regulated by the receptor α itself in hormone dependent fashion.

In another study Dr. Nikodem's group fully characterized two thyroid hormone response elements. Malic enzyme element functions as a thyroid hormone-receptor dependent enhancer of the GC rich malic enzyme promoter and the myelin basic protein element as an enhancer of the promoter containing TATA and CAAT boxes. There are thytoid hormone dependent quantitative differences in transcriptional activation with the receptor α and β on these two thyroid hormone response elements. So far, they demonstrated that both the myelin basic protein thyroid hormone response element (arranged as an inverted palindrome) and a TATA like sequence are required for more efficient thyroid hormone responsiveness elicited by the β receptors. In the case of the malic enzyme promoter, more efficient thyroid hormone dependent activation by the α receptor requires the malic enzyme element (arranged as a direct repeat) and an element within 122 nucleotides upstream from the start of transcription.

Dr. Nikodem and collaborators also constructed plasmids expressing the receptor α with polyhistidine tail Baculovirus and E. coli expression systems and a nickle column have been used to purify the overexpressed receptor. This receptor is being used to study various parameters in heterodimer formation of the receptor and other nuclear proteins and effect of the ligands in binding of these complexes to thyroid hormone response elements.

Dr. Sato and her colleagues are investigating functions of the POU domain gene family which has been shown to be important in tissue specific gene regulation during development. It has been proposed that POU class III transcription factors act in a combinatorial fashion to establish various neuronal phenotypes in the brain. The role of two of these POU class III factors, XLPOU 1 and XLPOU 2, in early neural development was studied. In situ hybridization analysis of *Xenopus* embryos has demonstrated that in the neural plate, XLPOU 1 gene expression is restricted to the future midbrain and hindbrain. In tailbud stage embryos, additional XLPOU 1 gene expression is observed in the forebrain and eyes. XLPOU 2 gene expression is observed in the ventral forebrain, midbrain, and hindbrain. XLPOU 1 and XLPOU 2 should prove to be useful markers in studying how the anterior part of the brain is established. When embryos are treated with retinoic acid (RA) or its derivatives, anterior-posterior polarity in the nervous system is disrupted, resulting in a posterization of the embryonic brain. Using lineage tracers to map the cell fate of anterior blastomeres, it was demonstrated that the underlying mechnism of a RA posteriorization of the brain was due to a change in cell fate. Furthermore, concurrent with the loss of forebrain structures that occurs with low doses of RA, cells in a more posterior position, the X1POU 1- and X1POU 2- expressing cells of the midbrain and eye, were shown to proliferate.

Endocrinology Section

Dr. Robbins has continued his studies on thyroid cancer. However, recruitment of high risk thyroid cancer patients for combination therapy with I-131 and low dose doxorubicin has been discontinued because the number of referrals has been insufficient to evaluate therapeutic effectiveness. Combined therapy is continuing with the 8 patients randomized to this treatment arm and toxicity will be evaluated and compared with controls who received I-131 alone. The toxicity data will be important for therapy recommendations.

MECHANISMS OF GENE REGULATION SECTION

SUMMARY

Dr. Nikodem and colleagues continue to study the mechanism of thyroid hormone action. They demonstrated that the 9-cis retinoic acid receptor, a general heterodimerization partner for thyroid hormone receptor, can further enhance thyroid hormone responsiveness only of some genes. They have shown that the thyroid hormone/thyroid hormone response element-inducible expression of the myelin basic protein gene is not further enhanced by the 9-cis retinoic acid receptor β-thyroid hormone receptor, heterodimer while this heterodimer dramatically enhanced expression of another target gene, malic enzyme. Furthermore, their studies revealed that the 9-cis retinoic acid receptor β reverses the thyroid hormone/thyroid hormone response element-dependent down-regulation mediated by the negative thyroid hormone response element found within the promoter of the mouse thyroid stimulating hormone gene. Thus, 9-cis retinoic acid receptor inhibition of the thyroid hormone dependent negative regulation offers a novel mechanism whereby thyroid hormone receptor negative regulation might be prevented.

Dr. Nikodem's group characterized a promoter of the thyroid hormone receptor α gene. Mutational analyses of the genomic sequences revealed that in addition to the 5' flanking sequences, regulatory elements in the first intron have the most significant effect on the receptor promoter activity. The three copies of AGG sequence function as an enhancer, while two octamer binding motifs are negative regulators. They proposed that a tissue specific expression of this gene depends on the relative levels of transcriptional factors binding to these intronic sequences.

In another study Dr. Nikodem and her coworkers identified proteins in a rat brain and liver nuclear extracts interacting differentially with different thyroid hormone response elements and thyroid hormone receptors α and β. They showed that the interaction between the brain nuclear protein and thyroid hormone receptor on myelin basic protein thyroid hormone response element is highly specific, being strictly β–isoform and thyroid hormone response element dependent. This nuclear factor is expressed only in brain and during a limited developmental phase (from 19 day embryo to 15 day neonatal) when overall brain maturation and the myelin basic protein gene expression are sensitive to thyroid hormones. Molecular cloning of this factor has been initiated.

On a different project Dr. Nikodem's coworkers have extended their studies on identification of two novel thyroid hormone response elements. They showed that the proteolipid protein gene contains a thyroid hormone response element consisting of a direct repeat separated by two nucleotides, in contrast to a classical thyroid hormone response element where four nucleotides separate two half sites. Consequently, this element is not further activated by thyroid hormone receptor-9-cis retinoic acid receptor heterodimer but thyroid hormone responsiveness depends on the formation of a novel heterodimer containing the receptor β and peroxisome proliferator activated receptor. The critical region of thyroid hormone receptor β consists of three amino acids in the DNA binding domain.

Lastly they showed that viral promoters can also contain thyroid hormone response elements, which are functional. Such an element was discovered in the Simian Virus late promoter at the major late transcription start site. The role of this element in the viral cycle, if any, remains to be elucidated.

Dr. Sato's laboratory continues to study effects of a family of POU-domain gene products on anteroposterior axis formation during amphibian embryo genesis. They investigated the role of two of these POU class III factors, *XlPOU1* and *XlPOU 2,* in early neural development. They used *XlPOU 1* as a molecular marker of the developing anterior nervous system, in addition to lineage mapping to study the action of retinoic acid on anteroposterior patterning in the embryo. They showed that with increasing doses of retinoic acid, the anterior structure of the brain were transformed progressively to more posterior ones. Additionally, with high doses of retinoic acid the normal cell fate of the A1 lineage was changed from a mostly neuronal phenotype to an epidermal one. To establish a functional role for *XlPOU 2* in embryonic development, they have misexpressed/overexpressed the *XlPOU 2* gene product by injecting synthetic *XlPOU 2* mRNA into specific blastomeres of the 32-cell stage embryo (A1 or A4 cells). As a result of this perturbation, embryos displayed secondary axes, eyes that were fused to the midbrain, abnormalities in cell fate.

Dr. Robbins has continued his studies on thyroid cancer. However, recruitment of high risk thyroid cancer patients for combination therapy with I-131 and low dose doxorubicin has been discontinued because the number of referrals has been insufficient to evaluate therapeutic effectiveness. Combined therapy is continuing with the 8 patients randomized to this treatment arm and toxicity will be evaluated and compared with controls who received I-131 alone. The toxicity data will be important for therapy recommendations.

The phase I/II study of recombinant human TSH in recently operated thyroid cancer patients has been completed and the results are being evaluated. Because of encouraging initial results a phase III study is now underway with patients in varying stages of their disease. This multi-institutional study is in collaboration with Bruce Weintraub, MCEB, and the Genzyme Corporation, and is expected to improve testing for residual or recurrent thyroid cancer.

In conjunction with development of epidemiological studies to evaluate the apparent increased incidence of thyroid cancer in children affected by the nuclear power plant accident in Chernobyl, a workshop was held at NIH to evaluate some clinical management problems that ensued. It was concluded that the aggressive behavior of the disease in these children is characteristic of thyroid cancer in this age group and not a result of radiation. It was also concluded that potential preventive therapy with L-thyroxine on a broad scale is not indicated at this time.

Drs. Robbins, Benvenga and Cahnmann continued their investigation of thyroxine (T_4) binding to apolipoproteins. The current work focused on purified apoE. Scatchard analysis revealed a single site with Kd ~33 nM and a greater affinity for T_4 than for T_3. By photoaffinity labeling, the T_4 site was localized to the N-terminal, exon 3-coded region (aa 1-62). It was also shown that various apolipoproteins differ in their sensitivity to lipids and drugs that inhibit T_4 binding, and that T_4 binding is affected when the apoprotein is incorporated in the lipoprotein particles.

Dr. Cahnmann (in collaboration with Dr. Y. Ito, NHLBI) determined the optimal conditions for the syntheses and use of the affinity labels N-bromoacetyl-3,3',5-triiodothyronine and N-bromoacetyl thyroxine for the carrier-free iodine-labeled reagents as well as for the nonradioactive compounds. This work led to the discovery of a novel principle of purification by countercurrent chromatography, "elution peak sharpening," allowing collection of pertinent compounds in only a few small eluate fractions which avoids the necessity of pooling and working up large volumes of eluate.

All trans and 13-cis retinoic acid (RA) are the only commercially available stereoisomers of RA. Only the former is available in radioactive form (^3H). Exposure to light for extended periods of time has been used in various laboratories to convert all trans RA to a complex mixture of isomers. Dr. Cahnmann found that photoisomerization can be achieved by flash irradiation for a few seconds and a well-separated peak of 9-cis RA was obtained by reverse-phase HPLC. Since other isomers are formed faster than 9-cis RA, the yield of 9-cis RA cannot be improved by prolonged irradiation.

These compounds are widely used in the study of thyroid hormone binding protein and receptors, but previous preparative methods were non-ideal.

The phase I/II study of recombinant human TSH in recently operated thyroid cancer patients has been completed and the results are being evaluated. Because of encouraging initial results a phase III study is now underway with patients in varying stages of their disease. This multi-institutional study is in collaboration with Bruce Weintraub, MCEB, and the Genzyme Corporation, and is expected to improve testing for residual or recurrent thyroid cancer.

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These compounds are widely used in the study of thyroid hormone binding protein and receptors, but previous preparative methods were non-ideal.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 DK 52008-14 GBB

PERIOD COVERED
October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gene Expression and Human Genetics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.D. Camerini-Otero	Chief	GBB, NIDDK	
Others:	R. Gardner	Clinical Associate	J. Yancey-Wrona	IRTA Fellow
	E. Angov	IRTA Fellow	G. Poy	Biologist
	M. Kim	Visiting Fellow	L. Pike-Buchanan	Biologist
	L. Ferrin	Research Associate	Oleg Voloshin	Visiting Associate
	S. Pati	IRTA Fellow	Haim Manor	Courtesy Associate

COOPERATING UNITS *(if any)*
Carol Camerini-Otero - Diabetes Branch, NIDDK
Victor Zhurkin and Robert Jernigan - Laboratory of Mathematical Biology, NCI

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION
Molecular Genetics Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF-YEARS:	PROFESSIONAL:	OTHER:
10.00	9.5	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In order to dissect the biochemical steps involved in genetic recombination we have chosen to focus on a key early step: strand exchange between homologous parental DNAs. *In vitro*, the product of this strand exchange reaction is a joint molecule composed a single-strand DNA joined to one end of a linear duplex DNA. We have established a new paradigm for this homologous pairing, in essence, that recombinases such as the *E. coli* recA protein can hybridize a single strand of any sequence and an intact duplex. That is, the three strands form a novel DNA triplex (R-form DNA) in which the third strand may include both purines *and* pyrimidines. Thermal denaturation of chemically substituted DNAs and chemical footprinting of R-form DNA confirm that the third strand in R-form DNA is in the major groove of the duplex. We have also been able to isolate synaptic complexes consisting of all the three strands and recA. These structures have been studied in detail and will continue to be the basis of additional structural investigations. The kinetics of formation of these complexes have been used as a model for the homology search process in the obligatory recombination events during meiosis. The data shows that the search for homology is a very fast step that is not rate-limiting and that this is followed by a very slow step involving conformationl changes of the protein and the DNA. The synaptic complexes have also been used to develop a method for the selective cleavage of human DNA (RecA-Assisted Restriction Endonuclease (RARE) cleavage); this method has been applied to map and clone large fragments of DNA close to the Huntington Disease gene and to excise the very ends of chormosomes, that is, telomeres. In addition, we have been successful in cloning and expressing the gene for a thermostable RecA homologue. This protein should be very useful for mechanistic studies and for several important applications in biotechnology. Finally, we have been able to define a 35 amino acid stretch of the *E. coli* RecA protein as the single-strand binding domain.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52011-08

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Toxins and DNA Repair in *Xenopus* Oocytes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Eric Ackerman	Senior Staff Fellow	GBB, NIDDK
Others:	Timothy M. Jenkins	Visiting Fellow	GBB, NIDDK
	Joshua D. Levin	IRTA	GBB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH: Genetics and Biochemistry Branch

SECTION: Molecular Genetics Section

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

I. Our previous work demonstrated that toxins α-sarcin, ricin, Shiga toxin and Shiga-like toxin all inactivate protein synthesis in cells by attacking a highly conserved region near the 3'-end of 28S ribosomal RNA. Furthermore, microinjection of modified deoxyoligonucleotides, ribonucleotides and ribozymes complementary to the same region of 28S RNA abolish protein synthesis. We later demonstrated that a variety of nucleases are as effective as these toxins at abolishing protein synthesis. Most nucleases hydrolyze all cellular RNA, one of these nucleases, angiogenin, selectively hydrolyzes tRNA, but not ribosomal or mRNAs, when injected into *Xenopus* oocytes.

II. During early development *Xenopus* replicates its DNA nearly as fast as *E. coli* in log phase. We showed oocytes are an excellent source of DNA repair activity. Pyrimidine dimer repair was demonstrated by microinjecting UV-irradiated plasmid DNA into oocytes or adding damaged DNA to an extract derived from oocytes. We have also studied DNA repair for alkylated and chemically modified DNA as well as DNA replication with our repair extract.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-839

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 52012-09 GBB

PERIOD COVERED
October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structure-Function Relationships of Lysosomal Enzymes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Richard L. Proia	Research Biologist	GBB, NIDDK
Others:	Debra Boles	IRTA	GBB, NIDDK
	Olivia Johnson	Summer IRTA	GBB, NIDDK
	Francine Norflus	Biologist	GBB, NIDDK
	Mark Pennybacker	IRTA	GBB, NIDDK
	Shoji Yamanaka	Visiting Associate	GBB, NIDDK

COOPERATING UNITS *(if any)*
Ruth Navon, Saphir Medical Center, Kfar-Sava, Israel

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION
Biochemical Genetics Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF-YEARS:	PROFESSIONAL:	OTHER:
4.25	4.0	0.25

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Tay-Sachs disease is a form of the G_{M2} gangliosidoses caused by mutations in the HEXA gene. We have disrupted the HEXA gene in the murine embryonic stem cell line, J-1, by gene targeting via homologous recombination. Two targeted cell lines were used to derive chimeric mice that transmitted the mutated allele to their offspring. Heterozygous mice were intercrossed to produce mice homozygous for the disrupted gene. The homozygous mice exhibit a complete deficiency of β-hexosaminidase A, the enzyme that is absent in Tay-Sachs disease.

β-Hexosaminidase exists as two predominant isozymes, A, a heterodimer of α and β chains and B, a homodimer of β chains. Each subunit carries a different active giving rise to a preferred spectrum of substrates degraded by the respective isozyme. For example, only the heterodimer is able to degrade G_{M2} ganglioside. We have constructed chimeric subunits composed of portions of α and β sequences in order to identify the regions responsible for substrate preference. By this strategy we have determined that the α subunit substrate recognition site is composed of discontinuous stretches of amino acid sequence from both the N-terminal and C-terminal portions of the polypeptide. This work may lead to the creation of a homodimeric enzyme that can degrade G_{M2} ganglioside and which may be useful for enzyme replacement and/or gene therapy in Tay-Sachs disease.

PHS 6040 (Rev. 1/84)
U.S. GOVERNMENT PRINTING OFFICE 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 52015-04 GBB

PERIOD COVERED
October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Studies of Protein-DNA Interactions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Peggy Hsieh	Expert	GBB, NIDDK
Others:	Tammy C. Tobin	IRTA	GBB, NIDDK
	Igor G. Panyutin	Visiting Associate	GBB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH: Genetics and Biochemistry Branch

SECTION: Molecular Genetics Section

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects
☐ (a1) Minors
☐ (a2) Interviews
☐ (b) Human tissues
☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

DNA branch migration, a process whereby two homologous DNA duplexes exchange strands, is an essential component of genetic recombination. The elementary step of branch migration is movement of the Holliday junction one base pair in either direction with equal probabilities; thus branch migration can be modeled as a random walk. We previously demonstrated that a single base mismatch is sufficient to block nonenzymatic branch migration. Since homologous recombination regularly involves the exchange of DNA strands between two similar but not identical duplexes, our result suggested that recombination proteins are required to facilitate branch migration through sequence heterology. In order to further characterize the branch migration step, we developed an assay to determine kinetic parameters of spontaneous branch migration.

We have measured the step time of branch migration, that is the time required for the Holliday junction to move one base pair, as a function of temperature and ionic conditions. The rate of branch migration is extremely sensitive to the presence of metal ions. Branch migration is three orders of magnitude slower in the presence of Mg2+ compared to Na+. In the absence of any metal ions, branch migration is even faster. We attribute the effect of metal ions on the rate of branch migration to the effect these metals have on the conformation of the four-armed Holliday junction. Subtle differences in the conformation of the junction can have a tremendous effect on the overall rate of branch migration. Our results establish that spontaneous branch migration in the presence of Mg2+ is too slow to account for heteroduplex formation in biological systems and underscore the importance of proteins in promoting branch migration during recombination.

We are also interested in characterizing eukaryotic enzymes involved in recombination and DNA repair. We have obtained a partial cDNA clone from chicken with extensive sequence homology to yeast and human type I DNA ligases. Since chicken B cells carry out homologous recombination at unusually high frequencies, we hope to create a type I ligase-deficient cell by gene targeting to assess the physiological function of type I ligases.

PHS 6040 (Rev. 1/84)
U.S. GOVERNMENT PRINTING OFFICE 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52016 GBB

PERIOD COVERED

October 1, 1992, to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thyroid hormone interactions with cells and proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: GBB, NIDDK	Jacob Robbins	Chief, Endocrinology Section
Others: GBB, NIDDK	Salvatore Benvenga	Courtesy Associate
GBB, NIDDK	Marcia Phyillaier	Biologist
GBB, NIDDK	Hans Cahnmann	Scientist Emeritus

COOPERATING UNITS (if any)

D. Rader, Division of Intramural Research, NHLBI; Y. Ito, Division of Intramural Research, NHLBI

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3	2.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Continuing investigation of thyroxine (T_4) binding to apolipoproteins focused on purified apoE. Scatchard analysis revealed a single site with Kd ~33 nM and a greater affinity for T_4 than for T_3. By photoaffinity labeling, the T_4 site was localized to the N-terminal, exon 3-coded region (aa 1-62). It was also shown that various apolipoproteins differ in their sensitivity to lipids and drugs that inhibit T_4 binding, and that T_4 binding is affected when the apoprotein is incorporated in the lipoprotein particles.

In improving the methods for preparing the affinity labels, N-bromoacetyl thyroxine and N-bromoacetyl-3,5,3'-triiodothyronine, a novel principle of purification by countercurrent chromatography was developed.

Flash irradiation has been used to convert all-trans retinoic acid to a mixture of isomers, which can then be purified by HPLC. Current attention is focused on purification of 9-cis retinoic acid which is not available commercially.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52017-01 GBB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies of Thyroid Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Jacob Robbins	Chief, Endocrinology Section	GBB, NIDDK
Others:	Taisheng Lee	Clinical Associate	GBB, NIDDK
	J. Desiree Pineda	Clinical Associate	GBB, NIDDK
	Marcia Phyillaier	Biologist	GBB, NIDDK

COOPERATING UNITS *(if any)*

J. Reynolds, Nuclear Medicine, CC; M. Merino, Laboratory of Pathology, NCI; C. Meyers, NCI; R. Alexander, Surgery Branch, NCI; D. Fraker, Surgery Branch, NCI

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3	2.5	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Recruitment of high risk thyroid cancer patients for combination therapy with I-131 and low dose doxorubicin has been discontinued because the number of referrals has been insufficient to evaluate therapeutic effectiveness. Combined therapy is continuing, however, with 8 patients randomized to this treatment arm and toxicity will be evaluated and compared with controls who received I-131 alone. The toxicity data will be important for therapy recommendations.

The phase I/II study of recombinant human TSH in recently operated thyroid cancer patients has been completed and the results are being evaluated. Because of encouraging initial results a phase III study is now underway with patients in varying stages of their disease. This multi-institutional study is in collaboration with Bruce Weintraub and the Genzyme Corporation, and is expected to improve testing for residual or recurrent thyroid cancer.

In conjunction with development of epidemiological studies to evaluate the apparent increased incidence of thyroid cancer in children affected by the nuclear power plant accident in Chernobyl, a workshop was held to evaluate some clinical management problems that ensued. It was concluded that the aggressive behavior of the disease in these children is characteristic of thyroid cancer in this age group and not a result of radiation. It was also concluded that potential preventive therapy with L-thyroxine on a broad scale is not indicated at this time.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52018-02

PERIOD COVERED

October 1, 1992, to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of thyroid hormone on synthesis of myelin basic protein

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	V. Nikodem	Senior Investigator
Others:	A. Farsetti	Visiting Fellow
	F. Bogazzi	Visiting Fellow
	D. Pineda	Clinical Associate
	B. Dozin-Quarto	Visiting Scientist

COOPERATING UNITS (if any)

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Mechanisms of Gene Regulation

INSTITUTE AND LOCATION

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3	3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Merged with number Z01 DK 52021-02 GBB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52019-01 GBB

PERIOD COVERED

October 1, 1992, to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular biology of thyroid hormone receptor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Vera Nikodem	Chief, Mech Gene Reg Section	GBB, NIDDK
Others:	Paul Hallenbeck	Staff Fellow	GBB, NIDDK
	Roland Lippoldt	Chemist	GBB, NIDDK
	Marcia Phyillaier	Biologist	GBB, NIDDK
	Josef Lazar	Visiting Fellow	GBB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Mechanisms of Gene Regulation Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.5	2	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1) The thyroid hormone-inducible expression of some genes has recently been shown to be enhanced by 9-*cis* retinoic acid receptor. This effect appears to be at least partially elicited by the ability of 9-*cis* retinoic acid receptor to heterodimerize with thyroid hormone receptor and enhance its binding to the *cis*-acting thyroid hormone responsive elements found within those genes. In the present study, we show that the thyroid hormone/thyroid hormone response element-inducible expression of the myelin basic protein gene is not enhanced by 9-*cis* retinoic acid receptor ß while expression of another target gene, malic enzyme, is further enhanced by addition of 9-*cis* retinoic acid receptor. We also demonstrate the 9-*cis* retinoic acid receptor ß reverses the thyroid hormone/thyroid hormone response element-dependent down-regulation mediated by the negative thyroid hormone response element found within the promoter of the mouse thyroid stimulating hormone gene. The ligand for 9-*cis* retinoic acid receptor ß, either alone or in combination with thyroid hormone, did not alter the transcription mediated by any thyroid hormone response element studied. We concluded that the capacity of 9-*cis* retinoic acid receptor to modulate thyroid hormone-dependent transcriptional regulation depends upon the nature of the thyroid hormone response element.

2) Using the 5′ and 3′ deletion mutants, containing the thyroid hormone receptor α genomic sequences located upstream of the start of translation, we identified 3 genomic regions important for the expression of the rat thyroid hormone receptor α gene: 1. -137 to -60 (relative to the major start site of transcription) 2. 3 copies of AGG sequence located immediately downstream of the exon-intron junction; both these regions contribute positively to the promoter activity. 3. this element in the intron consists of two octamer binding motifs. They function as negative regulators. Thus tissue specific expression of the thyroid hormone receptor α gene can be governed by the relative tissue specific abundance of transcription factors binding to these regions.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52020-01 GBB

PERIOD COVERED

October 1, 1992, to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of anteroposterior patterning in early frog development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Sheryl Sato	Senior Staff Fellow	GBB, NIDDK
Others:	Veena Agarwal	Visiting Associate	GBB, NIDDK
	Samir Witta	Visiting Fellow	GBB, NIDDK
	Song Huang	Visiting Fellow	GBB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Mechanisms of Gene Regulation Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.6	0.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The POU domain gene family has been shown to be important in tissue specific gene regulation during development. It has been proposed that POU class III transcription factors act in a combinatorial fashion to establish various neuronal phenotypes in the brain. We are investigating the role of two of these POU class III factors, *X1POU 1* and *X1POU 2*, in early neural development. We have used *X1POU 1* as a molecular marker of the developing anterior nervous system, in addition to lineage mapping to study the action of retinoic acid (RA) on anteroposterior patterning in the embryo. We observed that with increasing doses of RA, the anterior structures of the brain were transformed progressively to more posterior ones. Additionally, with high doses of RA the normal cell fate of the A1 lineage was changed from a mostly neuronal phenotype to an epidermal one. Our data support the hypothesis that exogenous RA or a closely related derivative, causes posterior transformations in the embryo. To establish a functional role for *X1POU 2* in embryonic development, we have misexpressed/overexpressed the *X1POU 2* gene product by injecting synthetic *X1POU 2* mRNA into specific blastomeres of the 32-cell stage embryo (A1 or A4 cells). As a result of this perturbation, embryos displayed 2° axes, eyes that were fused to the midbrain, abnormalities in the outgrowth of cranial nerves IX, X, and XI, and alterations in cell fate. Thus, these experiments suggest an important role for *X1POU 2* in brain development.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 DK 52021-01 GBB

PERIOD COVERED
October 1, 1992, to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mapping of triiodothyronine responsive genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Vera Nikodem	Chief, Mech Gene Reg Section	GBB, NIDDK
Others:	Fausto Bogazzi	Visiting Fellow	GBB, NIDDK
	Beatrice Desvergne	Visiting Fellow	GBB, NIDDK
	Beatrice Dozin	Visiting Associate	GBB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION
Mechanisms of Gene Regulation Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. In this report we have shown that the proteolipid protein gene contains a potential thyroid hormone response element consisting of a direct repeat separated by two nucleotides. It, however, can only be activated by thyroid hormone receptor ß in the presence of thyroid hormone and peroxisome proliferator activated receptor. Thyroid hormone receptor α is ineffective. The critical region of thyroid hormone receptor ß consists of three amino acids at positions 143 - 145 in the distal box of the DNA binding domain. This study shows that the two thyroid hormone receptors can regulate a different set of target genes by selective heterodimer formation recognizing specific thyroid hormone response elements.

2. Using a gel shift mobility and DNase I footprinting assays we identified a novel thyroid hormone response element in the Simian Virus 40 late promoter at the major late transcription start site. This thyroid hormone response element when placed upstream of the thymidine kinase promoter functions as a positive regulatory element in a strictly thyroid hormone and thyroid hormone receptor dependent fashion. However, there is a difference between whether this thyroid hormone response element is in cis or trans to thymidine kinase promoter. In cis, this element acts as a thyroid hormone dependent enhancer. By contrast, when this element is a part of the Simian Virus 40 late promoter, trans to the thymidine kinase promoter, it acts as a thyroid hormone and a receptor dependent inhibitor of thymidine kinase promoter activity.

3. We have identified certain proteins in a rat brain and liver nuclear extracts that can interact differentially with the thyroid hormone receptor depending on the structure of thyroid hormone response elements and the receptor isoform (α or ß). Their distinct developmental and tissue specific patterns of expression most likely contribute to the tissue- and development-specific regulation of genes responsive to thyroid hormones. Cloning of these factors is underway in order to support this hypothesis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 52022-01 GBB

PERIOD COVERED
October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Protein Entry into the Secretory Pathway

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Harris Bernstein	Senior Staff Fellow	GBB, NIDDK
Others:	Nancy Ulbrandt	IRTA	GBB, NIDDK

COOPERATING UNITS *(if any)*

LAB/BRANCH: Genetics and Biochemistry Branch

SECTION: Molecular Genetics Section

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have initated studies to understand in detail how newly synthesized proteins which must be secreted from cells or inserted into biological membranes are identified and directed to transport sites in the endoplasmic reticulum (in eukaryotic cells) or the cytoplasmic membrane (in prokaryotic cells). The project combines biochemical and genetic approaches to investigate the role of the mammalian signal recognition particle (SRP), the signal recognition particle receptor (SRP receptor), and their bacterial homologs in this process. Previous studies have indicated that SRP and the SRP receptor are key components of the cellular machinery responsible for the "initiation" phase of protein export. We expect that this project will help to solve a number of important questions about two fundamental cellular processes, protein sorting and protein folding. In addition, aspects of this project will provide insight into the regulation of multi-step pathways and the function of proteins that have broad substrate specificies.

A number of mutations have been introduced into a gene encoding the 54kd subunit of mammalian SRP (SRP54). This protein has been shown to recognize "signal sequences" that earmark proteins for entry into the endoplasmic reticulum. The mutations have been selected to alter either the signal sequence recognition properties of the protein or its GTPase activity. Analysis of the mutant proteins involving the use of established in vitro assays for signal sequence recognition and protein transport into the endoplasmic reticulum has been initiated. These experiments will help elucidate the mechanism whereby SRP54 recognizes a wide spectrum of signal sequence substrates and the exact role of its GTPase activity in regulating the access of proteins to the secretory pathway.

Studies have also been intiated to understand the function of homologs of SRP and the SRP receptor in E. coli. The ease of performing genetic experiments makes this organism attractive; moreover, studies of the function of SRP and the SRP receptor in an evolutionarily ancient species should yield important information about their function in all cells. We have initiated genetic experiments to firmly establish the role of these homologs in the protein export process. We have also begun to explore the possibility of establishing in vitro assays for protein export that are dependent upon the activity of the homologs. Finally, we are beginning to use biochemical methods to determine whether the SRP receptor homolog interacts with proteins that have previously been shown to play an important role in protein secretion.

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U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52023-01 GBB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genes ancestral to the thyroid/steroid receptor family

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Joseph E. Rall	Senior Scientist	GBB, NIDDK
Others:	Zdenek Kostrouch	Visiting Associate	GBB, NIDDK

COOPERATING UNITS *(if any)*

Zoological Station, Naples, Italy

LAB/BRANCH: Genetics and Biochemistry Branch

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF-YEARS:	PROFESSIONAL:	OTHER:
2	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have used degenerate primers in PCR of DNA from yeast and medusa to identify genes homologous to those in the thyroid/steroid family of nuclear receptors. We have cloned and partially sequenced five different genes from yeast that appear to be homologous to the thyroid/steroid family. An additional 20 have been identified which are almost identical in the primer region but are somewhat divergent elsewhere. Some of these will be cloned and sequenced. Preliminary experiments with medusa DNA show after PCR, bands on a gel, one of which is the right size for a zinc finger protein.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE 1991 0-854-939

Summary of Branch Activities

All four sections of the Digestive Diseases Branch are engaged in investigations of basic biologic processes leading to alteration in the function of gastrointestinal tissues and are attempting to apply this information to understand the pathophysiology of various disorders involving the liver, pancreas and gastrointestinal tract. All four sections are also involved in attempts to improve therapy of clinical disorders such as neoplasms associated with overproduction of gastrointestinal hormones, hepatitis and fulminent hepatic failure. The important results found in the various sections are briefly summarized with more detailed descriptions in the individual section reports.

Annual Report of the Liver Diseases Section, National Institute of Diabetes and Digestive and Kidney Diseases.

SUMMARY:

I. Studies of the Natural History and Treatment of Chronic Type B Hepatitis.

A cohort of patients with chronic type B hepatitis is being evaluated and followed prospectively to determine the long-term natural history of this common form of chronic liver disease. Selected patients have been entered into therapeutic trials in which antiviral or immunomodulatory agents are being administered. The effects of FIAU, a nucleoside analogue, are currently being evaluated in patients with chronic hepatitis B. Although this agent was found to have potent antiviral activitiy against hepatitis B, prolonged administration (for more than 1 month) was found to be associated with severe toxicity and trials of FIAU have been discontinued. A controlled trial of recombinant alpha interferon is underway in children with chronic hepatitis B. (AM Di Bisceglie, JH Hoofnagle, MW Fried, R Sallie, H Conjeervaram, M Beames, S Straus [NIAID]).

II. Studies of the Natural History and Treatment of Chronic hepatitis C (formerly: non A, non B hepatitis).

Patients with well documented chronic hepatitis C are being evaluated to determine the long-term natural history of this common form of chronic liver disease. A cohort of such patients has been recruited to evaluate experimental therapies. Earlier studies showed that alpha interferon was effective in lowering serum aminotransferases, decreasing serum levels of hepatitis C viral RNA and improving liver histopathology. Alpha interferon is now approved by the Food and Drug Administration for use in chronic hepatitis C.

A randomized, double-blind, placebo-controlled trial of ribavirin is currently being conducted to evaluate the effect of this agent in the therapy of chronic hepatitis C. To date, 32 of 58 patients in this trial have completed the study. Prolonged ribavirin administration was associated with significant improvement in serum aminotransferase activities and in hepatic lobular necrosis despite unchanged serum levels of HCV RNA.

Human materials (including serum, semen, saliva, liver tissue and white blood cells) are collected from patients with chronic viral hepatitis as part these studies. These materials are studied in the laboratory using techniques of molecular biology to assess viral factors that influence human disease. These characteristics are correlated with the disease and changes with therapy. (AM Di Bisceglie, JH Hoofnagle, MW Fried, LH Simpson, C Yurdaydin, MG Swain, H Conjeevaram, R Sallie. Not NIH: SM Feinstone, K Krawczynski).

III. Studies of the Opiate System in Cholestatic Liver Disease

The hypothesis that increased opioidergic neurotransmission contributes to the pathophysiology of cholestasis, and in particular to the pruritus associated with this syndrome continued to be tested. In a double blind placebo controlled trial of 29 patients with pruritus from cholestatic liver diseases, naloxone infusions were associated with decreased scratching activity and decreased perception of pruritus (as assessed by a visual analogue scale). The use of the oral opioid antagonist nalmefene had also been evaluated in 17 patients with this form of pruritus, and was found to be associated with decreased scratching activity, and visual analogue score (VAS). Based in these findings, a double-blind placebo controlled trial of nalmefene was conducted in 11 patients with the pruritus of cholestasis. Nalmefene wasassociated with a 75% reduction of scratching activity and an 86% reduction in the VAS. These findings further confirm the involvement of the opioid system in the pathogenesis of cholestaisis and suggest that nalmefene may be useful in the treatment of this form of pruritus. Adult cholestatic livers from rats with cholestasis secondary to bile duct resection express preproenkephalin (ppENK) mRNA in proliferating bile ductules and appear to make endogenous opioids *de novo* as suggested by positive immunohistochemical stains. To further study the status of the opioid system in cholestasis in the content of proenkephalin-derived endogenous opiods was measured in bile-duct resected livers and in sham control. Cholestatic livers had significantly higher concentration of endogenous opioids suggesting that indeed, they accumulate in the liver in cholestasis and most likely, synthesize them *de novo*. ppENK mRNA was sought in the livers of patients with PBC in baseline liver biopsies and during methotrexate treatment by use of a solution hybridization assay. No ppENK mRNA was found.

IV. Hepatic Encephalopathy

Ameliorations of hepatic encephalopathy (HE) (both clinical and electrophysiologic) have been induced in animals with fulminant hepatic failure (FHF) by benzodiazepine (BZ) receptor antagonists. Furthermore, Purkinje neurons from rabbits in HE due to FHF exhibited increased sensitivity to depression by agonists of the GABA/BZ receptor complex, including a BZ, and in contrast to control neurons, exhibited excitation when exposed to BZ receptor antagonists. These findings suggest that in HE due to FHF: (i) There is increased GABAergic tone; (ii) Blokading of BZ receptors can ameliorate HE, (iii) BZ receptor agonist may contribute to HE. Increased levels of 1,4-BZs have been demonstrated in the brain of models of HE and humans with FHF. The efficacy of BZ receptor ligands in ameliorating

HE in animal models does not appear to be dependent on their intrinsic activity but may be related to their affinity for BZ receptor subtypes in addition to the diazepam sensitive receptor. In vivo and in vitro studies indicate that the hepatocellular cytoprotective effects of the prostaglandin PGE_2 are mediated via activation of cAMP. (E.A. Jones, J. Vergalla, C. Yurdaydin, M.G. Swain, N.V. Bergasa, A.S. Basile, P. Skolnick, S.M. Paul, non NIH: R.B. Rothman).

V. Trials of Therapies for Primary Biliary Cirrhosis.

Primary biliary cirrhosis (PBC is a progressive liver disease believed to be of autoimmune nature. Its etilogy is unknown. It is characterized by progressive intrahepatic cholestasis as a result of ongoing non-suppurative destructive cholangitis that affecing small intrahepatic bile ducts. Several trials have been conducted to study the effect of immunosuppressants in PBC including methotrexate (MTX). Ten patients with symptomatic PBC were treated in an open label pilot study of oral MTX (15 mg/week). Two remain on therapy after 3 and 5 years respectively, while the drug was stopped in the remaining patients after 1 to 2 years because of apparent lack of effect. A trial that studies the effect of 7.5 vs 15 mg per week of oral MTX was started. Patients were randomized to receive one of the two doses and stratified according to the presence or absence of symptoms. 19 symptomatic and 10 asymptomatic patients have been entered in to this trial. Preliminary observations indicate that patients with advanced disease (stages III or IV) do not seem to respond to MTX. Patients with earlier stages (I, II and "early" III) appear to have a better rsponse with a decrease in the activity of serum aminotransferases and alkaline phosphatase and histological improvement. (J.H. Hoofnagle, N.V. Bergasa, A.M. Di Bisceglie, M.G. Swain, C. Yurdaydin, S.C. Chia, H. Conjeevaram, R. Sallie, M. Fried).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 53100-05 DDB

PERIOD COVERED

October 1, 1992, to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Identification and characterization of receptors for GI peptides

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.T. Jensen	Branch Chief	DDB, NIDDK
Others:	S.A. Mantey	Chemist	DDB, NIDDK
	V.A. Fishbeyn	Clinical Associate	DDB, NIDDK
	R. Benya	Clinical Associate	DDB, NIDDK
	M. Orbuch	Clinical Associate	DDB, NIDDK
	T. Kusui	Visiting Associate	DDB, NIDDK

COOPERATING UNITS *(if any)*

Tulane University, New Orleans, LA (D.H. Coy); VA Medical Center, Cincinnati, OH (R. Bell); Johns Hopkins School of Medicine, Baltimore, MD (T.H. Moran); George Washington University, Washington, DC (T.W. Moody); NCI, LBC, NIH (J. Battey).

LAB/BRANCH

Digestive Diseases Branch

SECTION

Gastroenterology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.1	2.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A. Identification of receptors for GI peptides.

1. In collaboration with J. Battey (NCI,LBL,NIH) a new receptor related to the GRP and NMB receptors was cloned, called BRS-3 (Bombesin related subtype 3). At present the endogenous ligand is unclear.

2. Endothelin receptors (Et_A and Et_B subtypes) were described in rat pancreatic acini. In contrast to other tissues, binding to these receptors was regulated by a PKC mechanism by other secretagogues.

B. Characterization of GI peptide receptors by development of specific antagonists.

1. In collaboration with Prof. D.H. Coy, Tulane University, the first class of specific antagonists for NMB receptors was discovered, substituted octapeptide analogues of somatostatin (SS). Some of these analogues also had high affinity for mu opioid and SS receptors, but structure-function studies demonstrate these can be disassociated suggesting specific high affinity NMB receptor antagonists can be developed in the future.

2. Structure-function studies using conformationally restricted amino acid substitutions on GRP were performed in collaboration with Prof. D.H. Coy (Tulane University) and provided evidence to support a putative folded conformation of the receptor bound Bn and the importance of the His^{12} in receptor activation.

3. In collaboration with Prof. D.H. Coy (Tulane University) a long acting GRP receptor antagonist, ([penta-fluoro-D-Phe^6]Bn (6-13)methyl ester) was discovered which should be useful for in vivo studies.

4. A potent, selective GRP receptor antagonist which can be radiolabeled was developed. This is the first described radiolabeled antagonist for this receptor with high enough affinity to be useful. It has greater selectivity, is not internalized and not affected by G-protein interaction as is the radiolabeled agonist.

5. The receptor for calcitonin gene-related peptide (GRP) was characterized by chemical cross-linking studies combined with enzymatic digestion on both gastric smooth muscle cells and pancreatic acini. It is a N-linked sialoglycoprotein, Mr 57,000, does not contain disulfide linked subunits or O-linked sugars and is of the same subtype in both tissues.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK53101-05 DDB

PERIOD COVERED

October 1, 1992, to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cellular basis of action of gastrointestinal peptides

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.T. Jensen	Branch Chief	DDB, NIDDK
	D.C. Metz	Senior Staff Fellow	DDB, NIDDK
	R.V. Benya	Clinical Associate	DDB, NIDDK
	Y. Kitsukawa	Visiting Fellow	DDB, NIDDK
	J. Mrozinski, Jr.	Chemist	DDB, NIDDK

COOPERATING UNITS *(if any)*

Department of Biochemistry, George Washington University (T.W. Moody); National Institute of Dental Research, Clinical Investigations Branch, NIH (R.J. Turner); Department of Surgery, University of Cincinnati (R. Bell).

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.6	2.6	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

I. Actions of somatostatin (SS) and PACAP-related peptides on chief cells.

(1). We demonstrated for the first time that chief cells possess high affinity SS receptors likely of the $SSTR_1$, subtype by binding studies. Receptor occupation was regulated by agents that activate PKC or adenylate cyclase. SS receptor activation decreased activation of adenylate cyclase but had no effect on pepsinogen release by various secretagogues. (2). ^{125}I-PACAP bound with high affinity to receptors on chief cells. Binding studies showed ^{125}I-PACAP bound with high affinity to VIP receptors and low affinity to secretin receptors. Forty percent of the maximal ability of PACAP to stimulate pepsinogen release was due to occupation of VIP receptors and 60% to secretin receptors. II. Role of calcium in secretagogue-stimulated secretion from pancreatic acini. Using thapsgargin (TG), BHQ and cyclopiazonic acid (CPA) sustained enzyme secretion by secretagogues that increase IP_3 (1,4,5) was shown not due to an increase $[Ca^{2+}]_i$ per se, however, potentiation was. III. Cellular basis of action at NMB receptors. Using C-6 glioblastoma cells and NMB-receptors transfected into Balb 3T3 cells, NMB-R activation was shown to activate PLC, increase $[Ca^{2+}]_i$ and IP_3 and not to activate adenylate cyclase. The transfected receptor and native NMB-R functioned identically in regard to kinetics of binding, stoiochiometry, internalization, coupling to G proteins and activation of PLC suggesting these cells will be useful to explore ligand receptor interactions and molecular biological studies of receptor structure function. IV. Role of CCK receptors in experimental pancreatic cancer tumorigenesis. In collaboration with R.H. Bell, Dept. of Surgery, U. of Cincinnati, School of Medicine, overexpression of high affinity CCK receptors in premalignant and malignant tumors was shown and it was proposed this may result in a growth advantage for these tumors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53200-02 DDB

PERIOD COVERED

October 1, 1992, to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Management of islet cell tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.T. Jensen	Branch Chief	DDB, NIDDK
Others:	D.C. Metz	Senior Staff Fellow	DDB, NIDDK
	V.A. Fishbeyn	Clinical Associate	DDB, NIDDK
	D.B. Strader	Clinical Associate	DDB, NIDDK
	M. Orbuch	Clinical Associate	DDB, NIDDK

COOPERATING UNITS *(if any)*

National Cancer Institute, Surgery Branch, NIH (J.A. Norton, D. Fraker); Radiology Department, Clinical Center, NIH (J.L. Doppman); Pathology, NCI (Dr. Stetler-Stevenson).

LAB/BRANCH

Digestive Diseases Branch

SECTION

Clinical Investigation Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.1	3.1	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A. Control of gastric acid hypersecretion in patients with Zollinger-Ellison syndrome (ZES).

1. The recommended maintenance doses of Na^+-K^+ ATPase inhibitors was shown to be too high and by using lower doses savings of up to $6,000/year in medication expenses can be obtained.

2. An analysis of 10-years of continuous treatment with omeprazole in patients with ZES was reported. It was concluded the drug is safe and effective even with prolonged usage.

3. Lansoprazole, a new H^+-K^+ ATPase inhibitor was shown to be safe and effective and have a prolonged duration of action, therefore will be useful in patients with ZES.

4. Postcurative resection patients with ZES had a marked decrease in basal acid secretion, however 67% remained mild hypersecretors and will require continued low dose treatment.

B. Studies related to tumor localization, surgery, treatment of advanced disease.

1. In a prospective study it was shown that both fasting gastrin levels and secretin provocative tests need to be done to predict cure. Imaging studies and calcium provocative studies are not necessary.

2. Gastric carcinoid tumors were shown to be best identified by fine needle aspiration, rather than biopsy in hypergastrinemic patients.

3. In a collaborative study with the Pathology Department (NCI) flow cytometry results of gastrinomas were shown to correlate independently with the disease extent.

4. Prospective studies with the Radiology Department demonstrated that MRI was now the best noninvasive method to localize metastatic gastrinoma to the liver in patients with ZES. The use of intra-arterial secretin with gastrin sampling was shown to be superior to portal venous sampling to localize gastrinomas.

5. A prospective study with the NCI Surgery Branch, demonstrated that duodenotomy was required to localize primary duodenal gastrinomas.

6. Interferon was shown to have limited efficacy in the treatment of metastatic gastrinomas.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 53201-04 DDB

PERIOD COVERED

October 1, 1992, to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Receptors on gastric smooth muscle cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.T. Jensen	Branch Chief	DDB, NIDDK
Others:	T. Pradhan	Chemist	DDB, NIDDK
	Z-F. Gu	Visiting Fellow	DDB, NIDDK
	Y. Kitsukawa	Visiting Fellow	DDB, NIDDK

COOPERATING UNITS *(if any)*

Peptide Research Lab, Tulane University, (Drs. Rossowski and Coy).

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

I. Role of protein kinase A (PKA) in mediating smooth muscle relaxation. Using the competitive antagonist of the action of cAMP, R-p-cAMPs, activation of PKA was shown to be primarily responsible for mediating smooth muscle relaxation produced by adrenergic agents and various neuropeptides.

II. Gastric smooth muscle cells possess two classes of endothelin receptors, however, only one alters contraction. Gastric smooth muscle cells were shown to possess both ET_A and ET_B receptors, however in contrast to the pancreatic cells, they were not regulated by agents that activate PLC. Only occupation of the ET_A receptor altered contractile behavior.

III. Gastric smooth muscle cells possess high affinity galanin receptors which mediate relaxation by activating adenylate cyclase. Galanin was shown to have a direct affect on gastric smooth muscle cells cause relaxation. By binding studies, high affinity galanin receptors were characterized, occupation of which by agonists activated adenylate cyclase. Structure function studies showed the N-terminus of galanin determines receptor affinity and that this affinity is regulated by guanine nucleotide binding proteins.

IV. Chimeric galanin analogues are not receptor antagonsists. To investigate whether the two recently described classes of galanin receptor antagonists would be generally useful for investigating its complex effects on gut functions, their ability to alter smooth muscle function was investigated. In collaboration with Drs. Rossowski and Coy (Tulane University), these analogues were found to function as full agonists in both jejunal muscle strips and isolated gastric muscle cells, suggesting they will not be useful to investigate this peptide's role in motility.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53202-02 DDB

PERIOD COVERED

October 1, 1992, to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular characterization of receptors for GI peptides

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.T. Jensen	Branch Chief	DDB, NIDDK
Others:	S.A. Wank	Senior Investigator	DDB, NIDDK
	J. Pisegna	Clinical Associate	DDB, NIDDK
	T. Honda	Visiting Associate	DDB, NIDDK
	A. de Weerth	Visiting Fellow	DDB, NIDDK
	R. Benya	Clinical Associate	DDB, NIDDK
	S. Pope	Microbiologist	DDB, NIDDK

COOPERATING UNITS *(if any)*

K. Huppi, LG, MGS, NCI; J.F. Battey, LBC, DTP, DCT, NCI.

LAB/BRANCH

Digestive Diseases Branch

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.7	5.7	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A. Molecular characterization of NMB and GRP receptors.

1. We demonstrated that multiple serines and threonines located within the carboxyl terminus of the GRP receptor (GRP-R) distal to Cys^{341}, including but limited to those within the PKC consensus, specifically regulate GRP-R internalization.

2. In collaboration with J. Battey (NCI) we demonstrated that the Ile^{216} of the 5th transmembrane area of the NMB-R is critically important for determining selective high affinity NMB-R binding.

B. Molecular characterization for the CCK-gastrin related peptides.

1. Cloning of human CCK_A and CCK_B receptors. Both the human CCK_A and CCK_B receptors were cloned for the first time.

2. Mapping of $CCK_A$and CCK_Breceptors in rat brain. Using ^{35}S-labeled cRNA antisense probes the expression of CCK_A and CCK_B receptors was mapped for the first time.

3. Characterization of CCK_A receptors in gallbladder and pancreas. The CCK_A receptors from GB and pancreas were cloned from guinea pig and shown to be identical. These results do not support a previous proposal that they might represent different subtypes based on binding studies.

C. Molecular characterization of PACAP-receptors. For the first time the specific high affinity receptor for PACAP was cloned and expressed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 53516-02

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Opiate System in Cholestatic Liver Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A.M. DiBisceglie	Chief	LDS, NIDDK
Others:	N.V. Bergasa	Senior Staff Fellow	LDS, NIDDK
	J. Vergalla	Chemist	LDS, NIDDK
	M.G. Swain	Guest Researcher	LDS, NIDDK
	C. Yurdaydin	Visiting Associate	LDS, NIDDK
	R. Sallie	Visiting Associate	LDS, NIDDK

COOPERATING UNITS (if any)

NHLBI (Dr. S. Sabol, Dr. W. Scott Young), DIR, NIAID (Dr. David Alling), Biomedical Enginering (Thomas Talbot and Eli Walker)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The hypothesis that increased opiodergic neurotransmission contributes to the pathophysiology of cholestasis, and in particular to the pruritus associated with this syndrome was tested. In a double-blind placebo controlled trial of 29 patients with pruritus from cholestatic liver diseases, naloxone infusions were associated with decreased scratching activity and decreased perception of pruritus (as assessed by a visual analogue scale). The use of the oral opioid antagonist nalmefene had also been evaluated in 17 patients with this form of pruritus, and was found to be associated with decreased scratching activity and visual analogue score(VAS). BAsed on these findings, a double-blind placebo controlled trial of nalmefene was conducted in 11 patients with the pruritus of cholestasis. Nalmefene was associated with a 75% reduction of scratching activity and an 86% reduction in the VAS, cholestasis and suggest that nalmefene may be useful in the treatment of this form of pruritus. Adult cholestatic livers from rats with cholestasis secondary to bile duct resection express preproenkephalin (ppENK) mRNA in the proliferating bile ductules and appear to make endogenous opioids de novo. to further study the status of the opioid system in cholestasis the content of proenkephalin-derived endogenous opioids was measured in bileduct resected livers and in controls.

Cholestatic livers had significantly higher concentration of endogenous opioids suggesting that they accumulate in the liver in cholestasis and most likely, synthesize them de novo. ppENK mRNA was sought in the livers of patients with PBC in baseline liver biopsies and during methotrexate treatment by the use of a solution hybridization assay. No ppENK mRNA was found

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 53501-17

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies Relating to Pathogenesis of Hepatic Encephalopathy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A.M.DiBisceglie	Chief	LDS, NIDDK
Others:	J. Vergalla	Chemist	LDS, NIDDK
	M.G. Swain	Guest Researcher	LDS, NIDDK
	C. Yurdaydin	Visiting Associate	LDS, NIDDK
	N.V. Bergasa	Senior Staff Fellow	LDS, NIDDK

COOPERATING UNITS (if any)

Lavoratory of Neuroscience, NIDDK(P.Skolnick and A.S. Basile), Mental Health, DIR, NIMH(S.M. Paul), National Institute of Drug Abuse, Baltimore (R.B. Rothman)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Both clinical and electrophysiologic (VER waveform) ameliorations of hepatic encephalopathy (HE), have been induced in animals with FHF by benzodiazepine (BZ) receptor ligands with antagonist properties. Furthermore spontaneous in vitro activity of Purkinje neurons from rabbits in HE due to FHF exhibited increased sensitivity to depression by agonists of the GABA/BZ receptor complex, including a BZ, and in contrast to control neurons, exhibited excitation when exposed to BZ receptor antagonists. In addition a BZ receptor antagonist reversed the hypersensitivity of HE rabbit neurons to depression by a GABA agonist. The functional status of the chloride ionophore of the GABA/BZ receptor complex has been shown to be normal in a rat model of HE due to FHF. Radioligand binding to BZ receptors, determined autoradiographically, was decreased in thin unwashed sections from HE rabbit brains. Purification and characterization of HE rat brain extracts revealed the presence of reversible, competitive, BZ receptor ligands with agonist properties. Two of the ligands have been chemically characterized as the 1,4-BZs diazepam and N-desmethyldiazepam. The concentrations of the compounds were 2-9 fold greater in HE rat brain than control brain. Overall, these findings suggest that in HE due to FHF: (i) There is increased GABA-ergic tone; (ii)Blockading of BZ receptors can ameliorate HE; (iii) BZ receptor antagonists may be of value in the management of HE; and (iv) Endogenous BZ receptor agonists probably contribute to HE. The efficacy of BZ receptor ligands in ameliorating HE in animal models does not appear to depend on their affinity for BZ receptor subtypes in addition to the dizawpam sensitive receptor.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-DK 53503

PERIOD COVERED

October 1, 1992 though September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunologic Studies of Primary Biliary Cirrhosis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J. Vergalla	Chemist	LDS, NIDDK
	N.V. Bergasa	Senior Staff Fellow	LDS, NIDDK
	M. Shindo	Guest Researcher	LDS, NIDDK
	R. Sallie	Medical Staff Fellow	LDS, NIDDK

COOPERATING UNITS (if any)

Division of Gastroenterology, Univ. of Maryland of Baltimore

LAB/BRANCH

Digestive Disease Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 53511-12

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Trials of Therapies for Primary Biliary Cirrhosis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.H. Hoofnagle	Director,	DDDN,NIDDK
Others:	N.V. Bergasa	Senior Staff Fellow	LDS, NIDDK
	A.M. DiBisceglie	Chief	LDS, NIDDK
	M.G. Swain, S.C. Chia	Guest Researcher	LDS, NIDDK
	H. Conjeevaram	Guest Researcher	LDS, NIDDK
	R. Sallie	Visiting Associate	LDS, NIDDK
	C. Yurdaydin	Visiting Associate	LDS, NIDDK

COOPERATING UNITS (if any)

Laboratory of Experimental Immunology, NCI, Dr. David Adams

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.3	3.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Primary biliary cirrhosis (PBC) is a progressive liver diseases believed to be of autoimmune nature. Its etiology is unknown. It is characterized by progressive intrahepatic cholestasis as a result of ongoing non-suppurative destructive cholangitis that affecting small intrahepatic bile ducts. Several trials have been conducted to study the effect of immunosuppressants in PBC including methotrexate (MTX). Ten patients with symptomatic PBC were treated in an open label pilot study of oral MTX (15 mg/week). Two remain on therapy after 3 and 5 years respectively, while the drug was stopped in the remaining patients after 1 to 2 years because of apparent lack of effect. A trial that studies the effect of 7.5 vs 15 mg per week of oral MTX was started. Patients were randomized to receive one of the two doses and stratified according to the presence or absence of symptoms. 19 symptomatic and 10 asymptomatic patients have been entered in to this trial.

Preliminary observations indicate that patients with advanced disease (stages III or IV) do not seem to respond to MTX. Patients with earlier stages (I,II and "early" III) appear to have a better response with a decrease in the activity of serum aminotransferases and alkaline phosphatase and histological improvement.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-54001-02

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Natural History and Treatment of Chronic Type B Hepatitis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Adrian M. DiBisceglie	Chief	LDS, NIDDK
Others:	J. H. Hoofnagle	Director	DDDN, NIDDK
	M.W. Fried, H. Coonjeevaram	Staff Fellow	LDS, NIDDK
	R. Sallie	Visiting Assoc.	LDS, NIDDK
	M. Battegay, A. Mangia	Guest Researcher	LDS, NIDDK
	M. Shindo, Y-W. Chung	Guest Researcher	LDS, NIDDK
	M. Beames	Lab Technician	HSS, NIDDK

COOPERATING UNITS (if any)

E. Tabor, DCE, NCI, S. Straus, LCI, NIAID. J. Gerin, Georgetown Univ. S.M. Feinstone, CBER, FDA.

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.8	5.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A cohort of patients with chronic type B hepatitis is being evaluated and followed prospectively to determine the long-term nautral history of this common form of chronic liver disease. Selected patients have been entered into therapeutic trials in which antiviral or immunomodulatory agents are being administered. Several agents have been evaluated including alpha interferon, ribavirin, and fluoro-iodo arabinofuranosyl-uracil (FIAU).

Alpha interferon continues to be evaluated in atypical or unusual patients who would ordinarily excluded from controlled trials including those with decompensated cirrhosis, extra-hepatic complications of hepatitis B and atypical serologic markers. A controlled trial of interferon in children with hepatitis B is underway. To date, xx patients have been enrolled. None have completed the study.

Two pilot studies of FIAU, a nucleoside analogue, have been conducted. In the first, dose-finding, study 24 patients were treated FIAU at doses of 0.05, 0.1, 0.25 and 0.5 mg/kg/day orally for 4 weeks. FIAU therapy was associated with marked suppression (85% decrease) of serum HBV DNA levels after 4 weeks of therapy. Levels of HBV DNA often remained suppressed for prolonged periods after stoping FIAU. Two patients cleared hepatitis B e antigen from serum; hepatitis subsequently relapsed in one of these patients. In a second study of FIAU, disigned for patients to receive therapy for 6 months,15 patients eneterede the study. Severe side effects of the drug became apparent in 7 patients after taking the drug for 8 to 12 weeks. These patients appeared to develop a Reye's-like syndrome which is currently being investigated further. This study was discontinued because of toxicity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-DK 54002-02

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Natural History and Treatment of Chronic Type C Hepatitis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Adrian M. DiBisceglie	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Director	DDDN, NIDDK
	M.W. Fried, H. Conjeevaram	Staff Fellow	LDS, NIDDK
	R. Sallie, M.G. Swain	Visiting Associate	LDS, NIDDK
	C. Yurdaydin	Visiting Associate	LDS, NIDDK
	M. Shindo, L. Simpson	Guest Researcher	LDS, NIDDK
	K. Mahaney, M. Battegay	Guest Researcher	LDS, NIDDK
	M. Beames	Lab Technician	LDS, NIDDK

COOPERATING UNITS (if any)

H.J. Alter, Dept. Transfusion Medicine, CC, NIH. S.M. Feinstone, CBER, FDA. K. Krawczynski, Hepatitis Branch, CDC

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.6	4.6	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Patients with well-documented chronic hepatitis C are being evaluated to determine the long-term natural history of this common form of chronic liver disease. A cohort of such patients are available to evaluate experimental therapies. Previous studies have shown that alpha interferon therapy has a short-term beneficial effect in approximately 50% of sustained in approximately 20% after stopping interferon. A randomized, placebo-controlled trial of ribavirin is currently underway. So far, 32 of 58 patients have completed the first year of the study. Prolonged ribavirin administration was assoicated with significant improvement in serum aminotransferase activities and in hepatic lobular necrosis despite unchanged serum levels of HCV RNA.

ANNUAL REPORT OF THE

MOLECULAR AND CELLULAR ENDOCRINOLOGY BRANCH

(formerly, the MOLECULAR, CELLULAR AND NUTRITIONAL ENDOCRINOLOGY BRANCH)

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The MCEB continues basic and clinical investigations in the areas of molecular regulation and neuroendocrinology (Molecular Regulation and Neuroendocrinology Section, Bruce D. Weintraub, Chief); and growth and development (Growth and Development Section, Matthew M. Rechler, Chief). The Branch has had many visiting fellows and associates, as well as international collaborations with the University of Milan, Italy; University of Marseilles, France; Karolinska Institute, Sweden; Postgraduate School of Obstetrics and Gynecology, University of Auckland, New Zealand; University of Naples, Italy; Department of Medicine, University of Gothenburg, Sweden; University of Madrid, Spain. In the past year Dr. Weintraub was awarded the Commendation Medal of the United States Public Health Service for research accomplishments. He was also a plenary speaker at the Serono Symposium on Glycoprotein Hormones in Santa Barbara, California, the Symposium on Reproductive Endocrinology in Crystal City, Virginia, the Clinical Meeting of the AFCR/ASCI/AAP in Washington, DC, and the Endocrine Society Introduction to Molecular & Cellular Research Course in Orlando, Florida.

I. THYROTROPIN, THYROTROPIN-RELEASING HORMONE AND THYROID HORMONE: MOLECULAR BIOLOGY, REGULATION, ACTION, AND PATHOPHYSIOLOGY.

A. Production and Characterization of Recombinant Human Thyrotropin (TSH) and Its Analogs: Clinical Trials in Patients with Thyroid Cancer

1. Structure-function studies of rhTSH molecule: The role of sialylation in TSH bioactivity.

Terminal sialic acid and sulfates are of special physiological importance for human TSH and other glycoprotein hormones, because both residues provide net negative charge to the molecule and may influence the intrinsic activity, affect circulating half-life and consequently, biological activity *in vivo*. In the previous studies from our laboratory recombinant human TSH (rhTSH) has been used as a model to determine structure-function relationships of different isoforms of glycoprotein hormones. The highly purified rhTSH produced in a small hollow fiber bioreactor (rhTSH-N) as well as rhTSH commercially produced in a large-scale bioreactor (rhTSH-G), were quantitated by immunoassays, receptor binding assay and amino acid analysis and further characterized by a variety of physico-biochemical methods, including chromatofocusing and carbohydrate analysis. Compositional analysis of the fractions showed higher sialic acid content in the more acidic rhTSH-G fractions. Pituitary hTSH acidic isoforms showed higher total sulfate

and sialic acid content than the more basic fractions. The bioactivities of various TSH isoforms *in vitro* showed that the more basic and less sialylated fractions of rhTSH-G were more active than the more acidic fractions. In contrast to the *in vitro* data, highly sialylated and acidic rhTSH-G isoforms showed longer plasma half-life and a higher *in vivo* bioactivity than the basic forms. These results indicate that rhTSH, similar to the intrapituitary hTSH, exists as a mixture of charge isoforms which are related to the degree of sialylation. The degree of sialylation, highly dependent on the bioreactor production conditions, appears to be the major factor affecting the charge heterogeneity, metabolic clearance rate and bioactivity of rhTSH.

2. Biological and physico-chemical characterization of bovine pituitary TSH (bTSH): The role of sulfation in TSH bioactivity.

Both, rhTSH (sialylated not sulfated) and bTSH (sulfated not sialylated) constitute useful model to study the role of sialylation vs. sulfation in TSH bioactivity. Bovine pituitary TSH has been separated by chromatofocusing into several isoforms in the pI range 9.5-7.0. The more alkaline and less sulfated fractions of bTSH were more active, both *in vitro* and *in vivo*, than the more acidic and sulfated isoforms (based on quantitation of TSH by RIA). In contrast to the role of sialylation, the degree of sulfation does not significantly affect plasma half-life of bTSH isoforms. Therefore, the *in vivo* bioactivity of bTSH isoforms reflects differences observed *in vitro*. In contrast to the rhTSH, it is possible to predict the final *in vivo* potency of bTSH based solely on the *in vitro* bioactivity

3. Sequential enzymatic deglycosylation and resialylation of recombinant hTSH.

Recombinant (r)hTSH, expressed in Chinese hamster ovary cells contains sialic acid–terminating oligosaccharides as opposed to the presence of terminal sulfate residues in addition to the sialic acids in native pituitary hTSH. Sequential deglycosylation of hTSH using the exoglycosidases, neuraminidase, β-galactosidase and β-N-acetyl hexosaminidase increased the in vitro activity of the hormone more than 10-fold compared to the intact TSH. This is in contrast to the reports in literature on hCG, where progressive decrease in the in vitro activity was observed by sequential exoglycosidase digestion. The metabolic clearance of the derivatives was faster than that of intact hormone, but galactose-removed and N-acetylglucosamine-removed derivatives were cleared slower than sialic acid-removed rhTSH. However, the in vivo bioactivity (assessed by thyroxine production in response to TSH), decreased progressively with each monosaccharide removal and the N-acetylglucosamine-removed derivative showed minimal activity. Asialo rhTSH or incompletely sialylated rhTSH-N were resialylated using either recombinant α2,3 sialyl transferase (a kind gift of Dr. J.C. Paulson, Cytel) or α2,6 sialyl transferase. The increase in cyclic AMP stimulating activity due to sialic acid removal was reversed by resialylation of the terminal galactose residues. The resialylation also increases the in vivo bioactivities of the hormone. Thus, modification of the oligosaccharides by exoglycosidases and glycosyltransferases can be used as a powerful tool to delineate the functional role of carbohydrate in glycoproteins. Further, using this approach, it is possible to engineer more potent hormone analogs with longer half-life and/or higher bioactivity.

4. Structural determination of the oligosaccharides of rhTSH using Glycoprep and Glycomap.

Intact oligosaccharides were quantitatively isolated from rhTSH and purified by an automated hydrazinolysis method recently developed by Oxford Glycosystems. These oligosaccharides

were then labeled with ^3H at the reducing terminal by reduction with NaB^3H_4. The labeled oligosaccharides were desialylated by neuraminidase digestion and subjected to gel filtration under optimal conditions to fractionate them based on size. Coelution with a partial hydrolysate of dextran indicated that rhTSH oligosaccharides fractionate into at least four peaks of hydrodynamic volume corresponding to 22.03, 19.33. 16.44 and 13.52 glucose units. The latter two peaks possibly represent bi- and triantennary oligosaccharide chains with the presence or absence of fucose. These peaks are being further analyzed by using combinations of exoglycosidase digestions, the reagent array method to obtain the sequence data. These data indicate that the oligosaccharides of rhTSH are heterogeneous and are composed of mainly bi- and triantennary chains with minor populations of multiantennary chains.

5. Structural analysis of TSH by NMR spectroscopy and X-ray crystallography.

Experiments are under way to obtain the three dimensional structure of TSH using NMR and X-ray crystallographic techniques. In initial experiments, pituitary-derived TSH was found not to be suitable for obtaining NMR spectra due to its high heterogeneity and recombinant TSH although less heterogeneous was unsuitable due to its size. To date, the α subunit was found to give the best resolution on one dimensional spectral analysis. Removal of sialic acid from rhTSH did not improve the resolution. Structural determination of the subunits rather than the dimer appears to be a more practical way due to the size limitations at this time. To achieve this, recombinant TSH with carbon and nitrogen isotope labels will be produced. Subunits isolated from this labeled TSH will be combined with unlabeled complimentary subunits to obtain differentially labeled TSH. Then, 3-D NMR spectra can be measured on the dimer as well as on the individual subunits to obtain complete structural information.

6. Clinical trials of recombinant TSH in patients with thyroid cancer: Construction of a long acting analog of TSH

Recombinant human thyrotropin has been produced after transient or stable transfection of either an α cDNA or minigene and β minigene in various mammalian cell lines. Such TSH is biologically active in and is being tested in in patients with thyroid cancer. An Investigational New Drug Application has been approved by the Food & Drug Administration and phase 1 and 2 clinical trials have been completed at NIH and 4 other medical centers showing preliminary efficacy and safety in 19 patients. Phase 3 trials at NIH and 11 other medical centers involving over 100 patients are currently near completion. In the future recombinant TSH and its analogs are expected to be useful in stimulating uptake of radioactive iodide for diagnostic and therapeutic purposes and will eliminate the need for performing uptake studies in hypothyroid patients. Because of its therapeutic potential, a longer acting analog of TSH has been developed by fusing carboxy terminus extension peptide (CTEP) of hCGβ onto hTSHβ. Using the polymerase chain reaction (PCR), an hTSHβ minigene (989 bp) and a CTEP hCGβ DNA fragment (100 bp) were synthesized and a fusion gene was constructed by sequential cloning. Human embryonic kidney (293) and monkey kidney (Cos-7) cells were cotransfected with either pLBCMV.TSHβ (WT) or pLBCMV.TSHβ.CTEP hCGβ (chimera) with pAV2.hCGαcDNA. Both WT and chimeric TSH were expressed to the same extent as judged by immunoradiometric assay (IRMA) and chemiluminescence immunometric assay, suggesting that CTEP hCGβ had no adverse effect on αβ subunit assembly and/or secretion of TSH heterodimer. The bioactivities of WT and chimeric TSH were determined by their ability to stimulate cAMP production and ^3H thymidine uptake in rat thyroid FRTL-5 cells. Both WT and chimeric TSH expressed in 293 and

Cos-7 cells displayed similar *in vitro* bioactivity. However, the chimera displayed considerably longer half-life *in vivo* as well as enhanced *in vivo* bioactivity. Thus such analogs may prove advantageous as a second generation analog of recombinant thyrotropin.

. . . . N. R. Thotakura, M. Szkudlinski, L. Joshi, J. East-Palmer, B. D. Weintraub

B. Transcriptional regulation of the human TSHβ (hTSHβ) gene

The hTSHβ gene is expressed only in the thyrotrophs of the anterior pituitary, where its expression is induced by TRH, PMA or cAMP and inhibited by the thyroid hormone. Our previous studies indicated that the hormonal induction of the hTSHβ gene requires the pituitary-specific factor Pit-1 which binds to the -128/-61 region, while the thyroid hormone inhibition is mediated by thyroid hormone receptor binding to the sequences in the first exon.

We have characterized the functional properties of the TGGGTCA motif at -1/+6 of the hTSHβ gene that is similar to the consensus phorbol ester response element (TRE) or the consensus cyclic AMP response element (CRE). We have found that both protein kinases C and A as well as TRH share a common mediator which recognizes the TGGGTCA element in activating the hTSHβ promoter. Following stimulation by PMA, forskolin or TRH, the TGGGTCA-specific factor acted together with Pit-1 bound to the upstream -128/-61 sequence to mediate the induction of the hTSHβ promoter. The induction required that both factors bind to their own binding sites, but Pit-1 neither increased the binding of the TGGGTCA-specific factor to its target sequences nor associated with this factor to form a heterodimer. The TGGGTCA-specific factor was present in three cell lines tested and was composed of protein(s) immunologically related to c-Jun and c-Fos but not to CREB. By using the hTSHb reporter plasmids in which the TGGGTCA element was converted to consensus TRE or CRE motifs, we found that, within the context of the hTSHβ promoter, the TGGGTCA element is a more potent TRE or CRE than the consensus sequences. Based upon the results of this study, we propose a model in which the TGGGTCA-specific AP-1-like factor functionally cooperates with the tissue-specific factor Pit-1 to activate the hTSHβ gene.

In addition, we have localized a position and orientation-independent repressor element between -188 and -128 immediately upstream of the Pit-1 binding sites of the hTSHβ promoter. The negative effect of this element was not cell type specific. It decreased basal as well as inducible expression of the transfected hTSHβ gene in both the thyrotropic and nonthyrotropic cells. Part of this repression appears to be mediated by a transacting repressor which can be titrated away with an excess of the -188/-128 fragment. As suggested by the cell-independent negative activity, gel mobility shift analyses and DNase I footprinting assays revealed a protein(s) binding to the repressor element in all cell types tested. Furthermore, we have observed that the repressor element itself and the adjacent 5' upstream 400-500 bp sequences are highly A+T rich and have potential binding sites for topoisomerase II. In an in vitro binding assay using nuclear matrices prepared from HeLa and thyrotropic tumor cells, the A+T rich region acted as matrix attachment regions (MARs). Some MARs have been suggested to be involved in chromatin loop formation. Such a configuration may lock the gene promoter within the loop to turn off the transcription. We are currently studying whether the repressor activity is obtained through interaction with the nuclear matrix.

In summary, it appears that Pit-1 and the AP-1-like factor interact directly or indirectly resulting in the activation of the hTSHβ gene that is further regulated by the repressor element. The effect of the repressor is dominant over the positive regulatory effects of Pit-1 and the AP-1-like factor. How the thyrotropic cells overcome the dominant repressor activity to express the hTSHβ gene is under investigation.

The sequence from -1 to +6 bp in the hTSHβ gene contains overlapping putative thyroid hormone and AP-1 response elements. We have shown interaction between the AP-1 constituents c-fos and c-jun and thyroid hormone receptor in this region by transient transfection experiments using a -125 to +37 bp hTSHβ fragment. T3 inhibition was completely abolished by c-jun, but increased threefold by c-fos. A single transversion mutation at +2 bp restored T3 inhibition in the presence of c-jun and markedly reduced binding of purified c-jun by gel mobility shift assay. Thus, c-fos and c-jun influence T3 inhibition of hTSHβ expression in opposite direction acting through a response element shared with thyroid hormone receptor. Control of the relative cellular levels of these two proto-oncogenes may play a major role in modulating thyroid hormone inhibitory responses.

. . . . M. K. Kim, A. J. Mixson, L. A. Lesoon-Wood, D. L. Bodenner, B.D. Weintraub

C. Mutant β_1 T3 receptors from patients with generalized resistance to thyroid hormone: Molecular genetic, clinical and biochemical characterization.

We continue a large number of molecular genetic, clinical, and biochemical studies of kindreds with generalized resistance to thyroid hormone (GRTH) which have provided many insights into the molecular actions of thyroid hormone. This syndrome is characterized by high circulating levels of thyroid hormones, inappropriately normal or elevated serum TSH but variable degrees of tissue resistance within affected individuals as well as between individuals harboring identical mutations in the ligand binding domain of the T3-beta1 receptor gene. Studies of the transcriptional capacity of mutant receptors and their complex interactions with auxiliary proteins have demonstrated that mutant receptors inhibit the function of normal receptors by a dominant negative effect. This effect was dependent both on the type of mutation, which influenced the degree of homodimer or heterodimer formation, as well as on the nature of the thyroid hormone response element. Thus, it was greatest on the lap-TRE compared with the other two artificial TREs, pal and DR+4; that T3 had least effect in dissociating mutant homodimers bound to the lap-TRE and that RXR-beta did not overcome the dominant negative effects. Competition for DNA-binding rather than for limiting amounts of RXR-beta is the more likely mechanism mediating the dominant negative effects and these findings may account for the different tissue susceptibilities in GRTH. Naturally-occurring TREs in the promoter of various thyroid-responsive genes, e.g. alpha-myosin heavy chain, rat growth hormone , rat malic enzyme and myelin basic protein show certain differences compared to artificial TREs. We are currently in the process of characterizing the effects of mutant receptors on these more physiological TREs.

The wild type form of the both normal and several mutant receptors have been cloned into a vector which is expressed in a baculovirus/insect cell system. Thirteen positive clones appear to express the 50kDa. protein and a purification scheme has been developed to purify expressed receptors. These receptors, overproduced in a eukaryotic system, will be characterized with respect to structure and function in terms of phosphorylation and glycosylation, DNA and ligand binding capabilities, and transcriptional activities. We also intend to express enough for

structural studies using NMR and X-ray crystallography.

We have also continued detailed clinical investigation of 38 unrelated kindreds with GRTH including approximately 100 affected individuals and 100 unaffected siblings used as controls. These studies have shown for the first time that the mutant thyroid hormone receptors of GRTH cause attention deficit hyperactivity disorder, decreased intelligence, dyslexia, delayed growth as well as cardiac disorders. GRTH is the first primary behavioral disorder in man related to a characterized genetic defect.

. . . . R. Wong, A. J. Mixson, D. Seto, F. Davis, B. D. Weintraub

II. INSULIN-LIKE GROWTH FACTORS.

We have extended our studies designed to understand how insulin-like growth factor binding proteins (IGFBPs) modulate the biological actions of IGF-I and IGF-II. The IGFs occur in plasma, other extracellular fluids, and tissues complexed to one or more members of a family of 6 IGFBPs. The IGFBPs provide a mechanism for the subtle and versatile regulation of IGF action. The IGFBPs determine the bioavailability of the IGFs and may inhibit or potentiate their actions. To better understand the biological role and mechanism of action of the IGFBPs, we have characterized the proteins, isolated cDNA and genomic clones, and studied the regulation of gene expression. (1) Hepatic IGFBP-1 transcription and IGFBP-1 mRNA levels are increased in streptozotocin-diabetic rats, and rapidly normalized following insulin treatment. Insulin also rapidly decreases IGFBP-1 transcription in H4-II-E rat hepatoma cells. Inhibition by insulin is dominant to stimulation by dexamethasone, cyclic AMP or phorbol esters. Ongoing protein synthesis is required for the rapid turnover of IGFBP-1 mRNA. (2) Both upstream and proximal sites are required for the efficient transcription of the TATA-less rat IGFBP-2 promoter. The proximal sites include 3 clustered GC boxes that bind Sp1 or closely related proteins, are sufficient to confer Sp1-induced promoter activity, and function synergistically to activate the promoter. (3) IGFBP-4 is the predominant IGFBP expressed by 3 bovine endothelial cell lines. Secreted IGFBP-4 and IGFBP-4 mRNA are increased by agents that increase intracellular cyclic AMP in a clonal endothelial cell line established from bovine parathyroid. (4) IGFBP-6 purified from human cerebrospinal fluid and rat IGFBP-6 from the rat PC12 pheochromocytoma cell line are O-glycosylated. Enzymatic deglycosylation does not affect the marked preferential binding affinity of either IGFBP for IGF-II versus IGF-I. The binding specificities of IGFBP-6 and the other IGFBPs for a panel of recombinant IGF-II mutants were distinct, but more similar to each other than to the IGF-II/Mannose-6-phosphate or IGF-I receptors.

. . . . L. A. Bach, Y. Boisclair, A. L. Brown, G. T. Ooi, D. Suh, L. Tseng, Y. Yang, C–Y, Lee, M. M. Rechler.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 DK 55000-21 MCEB

PERIOD COVERED
October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biosynthesis, Glycosylation, and Action of Thyrotropin: Clinical Trials of Recombinant TSH

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

PI:	B. D. Weintraub	Chief	MCEB, NIDDK
Others:	N. R. Thotakura	Visiting Scientist	MCEB, NIDDK
	M. Szkudlinski	Visiting Fellow	MCEB, NIDDK
	L. Joshi	Senior Staff Fellow	MCEB, NIDDK
	J. East-Palmer	Biologist	MCEB, NIDDK

COOPERATING UNITS *(if any)*
None

LAB/BRANCH: Molecular and Cellular Endocrinology Branch

SECTION: Molecular Regulation and Neuroendocrinology Section

INSTITUTE AND LOCATION: NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 3.5	PROFESSIONAL: 3.5	OTHER: 0

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects
[ ] (a1) Minors
[ ] (a2) Interviews
[ ] (b) Human tissues
[ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Recombinant thyrotropin has been produced by novel methods of transient or stable transfection of alpha and hTSH beta minigenes into Chinese hamster ovary cells. Utilizing products produced in a large scale bioreactor as well as a hollow fiber bioreactor, we have been able to purify several hundred milligrams of this product. Terminal sialylation of recombinant TSH determines in vitro and in vivo bioactivity as well as clearance rate. The carbohydrate structure of recombinant TSH has been characterized in detail and differs from pituitary TSH.

Through a Cooperative Research and Development Agreement with the Genzyme Corporation (Boston), recombinant human TSH is being used for clinical studies in patients with thyroid cancer. This product is expected to stimulate uptake of radioactive iodine for both diagnostic and therapeutic purposes and to obviate the need for performing uptake studies in hypothyroid patients. An Investigational New Drug Application has been approved by the Food and Drug Administration and phase I and II clinical trials have been completed at NIH and 4 other medical center showing preliminary efficacy and safety in 19 patients. Phase III trials at NIH and 11 other medical centers involving over 100 patients are currently near completion. Currently we have developed a second generation long acting TSH analog in which a chimeric beta subunit has been engineered.

PHS 6040 (Rev. 1/84)

U.S GOVERNMENT PRINTING OFFICE: 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 DK 55002-13 MCEB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of Pituitary Glycoprotein Hormones and Hypothalamic Releasing Hormones

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

PI:	B. D. Weintraub	Chief	MCEB, NIDDK
Others:	M. K. Kim	Senior Staff Fellow	MCEB, NIDDK
	A.J. Mixson	Senior Staff Fellow	MCEB, NIDDK
	L. A. Lesoon-Wood	IRTA Postdoctoral Fellow	MCEB, NIDDK
	D. L. Bodenner	Senior Staff Fellow	MCEB, NIDDK

COOPERATING UNITS *(if any)*

None

LAB/BRANCH: Molecular and Cellular Endocrinology Branch

SECTION: Molecular Regulation and Neuroendocrinology Section

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 2.5	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The human TSH beta gene is expressed only in the thyrotrophs of the anterior pituitary where it is regulated by hypothalamic hormones, cyclic AMP as well as thyroid hormone. Recent studies have indicated that TRH and cyclic AMP mediate their transcriptional action in large part through changes in phosphorylation of the specific pituitary factor pit-1 which binds to a region upstream of the start of transcription. In contrast, thyroid hormone mediates its inhibition through binding to a regulatory element downstream of the start of transcription in the first untranslated exon.

We have recently characterized in detail a TGGGTCA motif at -1/+6 of the hTSH-beta gene that mediates, in conjunction with the upstream pit-1 site, protein kinase C and A as well as TRH stimulation of hTSH-beta gene expression. We have also shown that c-fos and c-jun which interact with this downstream site modulate T3 inhibition of hTSH-beta gene expression.

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U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01DK55006-20MCE

PERIOD COVERED: October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Insulin-like Growth Factor Binding Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. M. Rechler	Chief, Growth & Development Section	MCEB, NIDDK
Others:	L.A. Bach	Visiting Associate	MCEB, NIDDK
	Y. Boisclair	Visiting Associate	MCEB, NIDDK
	A.L. Brown	Expert	MCEB, NIDDK
	G.T. Ooi	Visiting Associate	MCEB, NIDDK
	D. Suh	Visiting Fellow	MCEB, NIDDK
	L. Tseng	Chemist	MCEB, NIDDK
	Y. Yang	Sr. Staff Fellow	MCEB, NIDDK
	C-Y. Lee	Visiting Fellow	MCEB, NIDDK

COOPERATING UNITS (if any)
Molecular Biology Research Laboratory, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan (K. Sakano);University of Florence Medical School, Florence, Italy (M.L. Brandi);Centro de Investigaciones Biologicas, CSIC, Madrid, Spain (F. de Pablo)

LAB/BRANCH: Molecular & Cellular Endocrinology Branch

SECTION: Growth & Development Section

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 8.75	PROFESSIONAL: 7.75	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have extended our studies designed to understand how insulin-like growth factor binding proteins (IGFBPs) modulate the biological actions of IGF-I and IGF-II. The IGFs occur in plasma, other extracellular fluids, and tissues complexed to one or more members of a family of 6 IGFBPs. The IGFBPs provide a mechanism for the subtle and versatile regulation of IGF action. The IGFBPs determine the bioavailability of the IGFs and may inhibit or potentiate their actions. To better understand the biological role and mechanism of action of the IGFBPs, we have characterized the proteins, isolated cDNA and genomic clones, and studied the regulation of gene expression. (1) Hepatic IGFBP-1 transcription and IGFBP-1 mRNA levels are increased in streptozotocin-diabetic rats, and rapidly normalized following insulin treatment. Insulin also rapidly decreases IGFBP-1 transcription in H4-II-E rat hepatoma cells. Inhibition by insulin is dominant to stimulation by dexamethasone, cyclic AMP or phorbol esters. Ongoing protein synthesis is required for the rapid turnover of IGFBP-1 mRNA. (2) Both upstream and proximal sites are required for the efficient transcription of the TATA-less rat IGFBP-2 promoter. The proximal sites include 3 clustered GC boxes that bind Sp1 or closely related proteins, are sufficient to confer Sp1-induced promoter activity, and function synergistically to activate the promoter. (3) IGFBP-4 is the predominant IGFBP expressed by 3 bovine endothelial cell lines. Secreted IGFBP-4 and IGFBP-4 mRNA are increased by agents that increase intracellular cyclic AMP in a clonal endothelial cell line established from bovine parathyroid. (4) IGFBP-6 purified from human cerebrospinal fluid and rat IGFBP-6 from the rat PC12 pheochromocytoma cell line are O-glycosylated. Enzymatic deglycosylation does not affect the marked preferential binding affinity of either IGFBP for IGF-II versus IGF-I. The binding specificities of IGFBP-6 and the other IGFBPs for a panel of recombinant IGF-II mutants were distinct, but more similar to each other than to the IGF-II/Mannose-6-phosphate or IGF-I receptors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 DK55015-05 MCEB

PERIOD COVERED

October 1, 1992 to September 30, 199

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mutations of the Thyroid Hormone Receptor Gene in Patients with Thyroid Hormone Resistance

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

PI:	B.D. Weintraub, M.D.	Chief	MCEB, NIDDK
Others:	R. Wong	Visiting Associate	MCEB, NIDDK
	A. J. Mixson	Senior Staff Fellow	MCEB, NIDDK
	D. Seto	Senior Staff Fellow	MCEB, NIDDK
	F. Davis	Visiting Associate	MCEB, NIDDK

COOPERATING UNITS *(if any)*

Dr. Stephen J. Usala, Section of Endocrinology, East Carolina School of Medicine, Greenville, NC

LAB/BRANCH: Molecular and Cellular Endocrinology Branch

SECTION: Molecular Regulation and Neuroendocrinology Section

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 3.0	PROFESSIONAL: 3.0	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Generalized resistance to thyroid hormone (GRTH) is an inherited disease linked to mutations in the beta T3 receptor gene and characterized clinically by the resistance of peripheral and pituitary tissues to the action of thyroid hormone. Of the over 30 different mutations identified in the ligand binding domain of the beta receptor, several were shown to inhibit normal receptor function by a dominant negative mechanism which is likely to mediate the phenotype of this disease. Recent studies have indicated that this dominant negative effect is most likely mediated by competiton of mutant and normal receptor for binding to T3 response elements within various thyroid hormone responsive genes.

We have recently shown that natural thyroid hormone response elements (TRE's) show variation in the dominant negative effect compared to previously employed artificial TRE's. Both normal and several mutant receptors have been cloned into a vector which is expressed in a baculovirus/insect cell system. Such eukaryotic receptors are being characterized with respect to phosphorylation, DNA and ligand binding, transcriptional capability and structure. We have also continued detailed clinical studies of 38 unrelated kindreds comprised of approximately 100 affected individuals and 100 unaffected siblings used as controls.

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U.S GOVERNMENT PRINTING OFFICE: 1991 0-864-939

ANNUAL REPORT

THE LABORATORY OF MOLECULAR AND CELLULAR BIOLOGY

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The Laboratory of Molecular and Cellular Biology investigates the fundamental processes involved in genome replication and the control of gene expression, using both eukaryotic and prokaryotic systems. Our goal is to understand how these processes operate during normal growth and development, and are disrupted by disease.

The LMCB was reorganized this year following the departure of Dr. Barrie Carter, a leading investigator on adeno-associated virus, who had served as its chief. Nancy Nossal was appointed as the new chief, and the Section on Nucleic Acid Biochemistry, which includes the working groups of Dr. Nossal and Dr. Deborah Hinton, transferred from the Laboratory of Biochemical Pharmacology to LMCB.

In the Molecular Biology Section, the work on adeno-associated virus initiated by Dr. Carter is now being continued by the remaining members of the section, led by Roland Owens, a Senior Staff Fellow. Takami Oka directs the Section on Cell Growth and Differentiation, which uses the mammary gland as a model to study hormone-dependent, tissue-specific gene expression. The Steroid Hormone Section, under the leadership of S. Stoney Simons, focuses on the regulation of gene transcription by glucocorticoid hormones. Frank Tietze, in collaboration with William Gahl, NICHD, studies lysosomal transport and storage diseases. In the Nucleic Acid Biochemistry Section, Deborah Hinton directs work on bacteriophage T4 gene expression, and on a T4 encoded endonuclease related to that of the group I introns. Dr. Nossal's group is using a system of purified T4 proteins to study the enzymatic reactions governing DNA replication.

Function of DNA Virus Genomes in Animal Cells

The group led by R. Owens (formerly B. Carter's group) has continued studies of adeno-associated virus (AAV) as a model system for human DNA replication and gene regulation. AAV is also being developed as a gene delivery system with potential for use in gene therapy.

The Rep proteins encoded by AAV are involved in AAV replication and AAV gene regulation. Wild-type and mutant Rep proteins have been expressed from recombinant baculoviruses in insect cells, a rabbit reticulocyte transcription-translation system, or the HIV-1 long terminal repeat promoter in human 293 cell transient transfections. Regions important for several Rep protein functions have been identified. In particular, a domain has been identified within the amino-terminal portion of the Rep78 and Rep68 proteins which can direct binding to the AAV origins of replication (Owens/Weitzman/ Kyöstiö/Carter). They have also demonstrated the repression of AAV RNA levels by Rep proteins (Kyöstiö/Owens/Weitzman/Carter).

The preferential integration of AAV DNA into a region of human chromosome 19 is being investigated in collaboration with the B. Safer and R. Kotin and their colleagues (NHLBI). A binding site for Rep proteins has been identified near the preferred integration site (Weitzman/Owens/Kyöstiö). Further study of this phenomenon may lead to the development of AAV-based gene therapy vectors with site-specific integration.

In a project sponsored in part by the Cystic Fibrosis Foundation; T. Flotte, B. Carter and their co-workers (Afione/ Solow), in collaboration with the W. Guggino and P. Zeitlin (Johns Hopkins) and M. Drumm (U. Michigan) have developed an AAV-based delivery system for the cystic fibrosis transmembrane

conductance regulator (CFTR) gene. The group has corrected the chloride channel defect in a tissue culture cell line derived from lung cells of a cystic fibrosis patient. Dr. Flotte has now established his own laboratory at The Johns Hopkins University. Dr. Carter has left to become vice-president of Targeted Genetics Corporation.

Regulation of HIV by AAV

Another project of the Owens group is the study of the AAV Rep proteins as possible therapeutic agents for AIDS. Wild-type Rep proteins have been demonstrated in tissue culture to inhibit the replication of HIV-1, the etiological agent of AIDS. Their therapeutic potential is however limited because they also block cell division.

In collaboration with A. Rabson and B. Antoni (New Jersey Center for Advanced Biotechnology and Medicine), a series of mutant Rep proteins expressed from the HIV-LTR are being tested for their ability to block HIV-1 replication. Plasmids containing the mutant *rep* genes are co-transfected with an infectious proviral clone of HIV-1 into human cells and the cells and culture supernatants are assayed for reverse transcriptase activity, HIV-1 RNA and for HIV-1 gag protein.

The Owens group is concurrently attempting to produce cell lines making these mutant Rep proteins to determine which parts of the proteins are involved in the cell division block. The identification of a mutant Rep protein that blocks HIV-1 production without blocking cell division would be the basis for a novel treatment for AIDS.

Hormonal Regulation of Cell Growth and Differentiation

T. Oka's group in the Section of Cell Growth and Differentiation is studying the molecular mechanisms involved in hormone-dependent, tissue-specific gene expression using the mammary gland as a model. Currently his group focuses on elucidating the molecular mechanisms of steroid and polypeptide hormone interactions on casein gene expression and also studies the signal transduction pathway involved in prolactin stimulated transcription of milk protein genes.

Beta-casein gene expression can be induced by the synergistic actions of prolactin, glucocorticoid, and insulin, but is inhibited by progesterone. The regulatory sequence elements responsible for casein gene expression, have been elucidated by constructing casein-CAT chimeric genes and examining their expression in transient assays. These experiments suggest the existence of multiple regulatory elements in the 500 bp 5'-flanking region and corresponding binding factors which are responsible for hormonal regulation. One of these DNA binding proteins is found specifically in the mammary gland of pregnant mice, and this protein serves as a repressor that mediates the inhibitory action of progesterone on beta-casein gene transcription. This group has also identified and isolated another transcription factor, which appears to be involved in tissue-specific expression of the beta-casein gene.

In addition, a new project has been undertaken by this group to gain some insight into the molecular mechanism of prolactin action on milk protein gene expression. The cDNA clones for the two forms of prolactin receptor have been isolated from the mouse mammary gland and their sequences have been determined. The regulation of prolactin receptor gene expression and the function of two forms of prolactin receptor are being examined in order to elucidate the molecular pathway of signal transduction leading to milk protein gene expression.

Steroid Hormone Action

The mechanism by which glucocorticoid hormones regulate gene transcription is being examined by S. Simons' group in the Steroid Hormones Section. Such studies are of particular current relevance since steroid hormone receptors are arguably the best understood regulators of eukaryotic gene transcription. The steroid first diffuses through the cell membrane and binds to a receptor protein which, at least for glucocorticoid receptors, is predominantly cytoplasmic. The receptor-steroid complex then undergoes a process termed activation whereby the affinity of complexes for DNA and nuclei is dramatically increased. Once activated, the complexes bind to specific DNA sequences in the nucleus and, in an unknown manner, influence the transcription of specific genes in target cells.

They have found that the steroid binding domain of the glucocorticoid receptor not only binds steroid but also is sufficient for the association of a non-receptor protein, heat shock protein 90, which is required for steroid binding and whole cell localization of receptors. This result will greatly aid future molecular studies of the initial steps in steroid hormone action.

The nuclear binding of activated complexes is required for the expression of steroid responses and is different for glucocorticoid and antiglucocorticoid steroids. They have found that this binding cannot be reproduced *in vitro* using methodologies that work for other macromolecules. This suggests that novel processes are involved in glucocorticoid receptor binding to nuclei. Efforts to define these new processes, and thus permit future *in vitro* studies of nuclear binding, should be facilitated by the use of aurintricarboxylic acid, which they found readily distinguishes between receptors bound to DNA and non-DNA acceptor sites.

In vivo nuclear-bound activated receptor-steroid complexes often modulate the transcription of selected proteins in a tissue specific manner. Their finding of a new tissue selective element in the expression of a model glucocorticoid inducible gene represents a further advance towards identifying all of the components required for cell-free studies of tissue specific gene expression.

Collectively, their current findings contribute to the long term goal of defining the mechanism of steroid hormone action at a molecular level.

Lysosomal Transport and Storage Disease

This work is being conducted by Dr. Frank Tietze. Degradation of cellular biopolymers such as proteins and polysaccharides takes place chiefly within the lysosome. The end-products of lysosomal digestion of biopolymers are known to exit the lysosome through the agency of specific membrane carrier proteins. Defects in these carrier proteins can result in a number of inherited storage disorders which are characterized by intralysosomal entrapment of excessive amounts of specific metabolites such as cystine or sialic acid. Unlike the plasma membrane carrier proteins, many of which have been isolated and/or sequenced, nothing is known concerning the structure of the lysosomal membrane carriers.

Although direct purification of membrane transporters is hampered by the lack of a suitable assay system, such difficulty has been overcome in the past by the use of reconstituted lipid membrane systems or proteoliposomes, i.e. lipid vesicles incorporating the membrane proteins and which retain the original transport function. As a preliminary to employing this methodology for the purification of the lysosomal cystine carrier, lysosomal membrane vesicles have been prepared from rat liver and have been investigated with respect to their transport activity. Initial studies have indicated that such vesicles possess cystine carrier activity and may therefore serve as useful starting material for preparation of functional proteoliposomes.

Transcription initiation.

During infection, transcription of different classes of bacteriophage T4 genes is performed by the host RNA polymerase, which is modified by ADP-ribosylation and by the association of specific phage factors at middle and late times. Transcription from middle promoters additionally requires the T4 MotA protein. Deborah Hinton and her associates are investigating the mechanism of MotA action at the T4 middle promoter PuvsX. Like other *motA*-dependent promoters, PuvsX contains a match to the sequence ('MotA box') of (t/a) (t/a) TGCTT (t/c) A, centered at -30. In addition, other possible MotA boxes are found in PuvsX, centered at -35, -51, -70, and -87. Their DNase I footprint analyses indicate that MotA protects PuvsX in the region from -25 to -59. This includes the MotA box at -30, but also the farther upstream sites centered at -35 and -51. *In vitro* transcription of a template lacking the MotA box at -51 indicates that while this site is not required for activation, it contributes to MotA action under less favorable transcription conditions.

KMnO4 reacts specifically with unpaired thymidines and has been used to assay the unwinding of promoter DNA at the start of transcription. Using phage-modified RNA polymerase, they have found that MotA protein is required to produce KMnO4 reactive thymidines at the transcription start site of PuvsX. This result supports the idea that MotA protein acts at a step leading up to or at the unwinding of the promoter DNA by RNA polymerase.

The first 8 amino acids (aa) of MotA are replaced with 11 different aa in the MotA mutant protein Mot21. Although Mot21 binds PuvsX similarly to wild type MotA, it does not activate transcription. Furthermore, a proteolyzed C-terminal fragment of MotA, starting at aa 102, and a cloned C-terminal fragment of MotA, which starts at aa 105, also bind PuvsX DNA. These results identify the C-terminal half of the protein as the DNA binding domain and suggest that the N-terminal half may be involved in protein-protein (MotA-RNA polymerase?) interactions.

DNA Endonuclease.

Dr. Hinton and her colleagues discovered the T4 *segA* gene, and showed that it encodes a Mg++-dependent endonuclease that is related by sequence to a family of endonucleases present in group I introns. These intron endonucleases are responsible for the specific movement (homing) of the intron DNA into an intronless allele. Their characterization of the cleavage activity of the SegA protein indicates that it is active on circular or linear double-stranded DNA and on DNA containing unmodified cytosines or wild type T4 DNA containing hydroxymethylated, glucosylated cytosines. Linear cleavage products are ligated by T4 DNA ligase. SegA activity is stimulated by the addition of either ATP or ATPγS, suggesting that ATP binding but not hydrolysis is needed for this stimulation. Although SegA prefers to cleave at certain sites, their analysis of highly preferred sites has not revealed a simple consensus sequence at the site of cleavage. Furthermore, they find that as the concentration of protein or incubation time is increased, any DNA is digested. This hierarchy of site preference means that the apparent specificity of SegA is governed by the level of the protein, the length of incubation, and the presence of any factors that stimulate (such as ATP) or by the presence of DNA ligase which can reverse the reaction.

Mechanism of DNA Replication

The group led by Nancy Nossal is continuing their study of the enzymatic reactions necessary for DNA replication using *E. coli* bacteriophage T4 as a model system. Efficient DNA replication *in vitro* requires at least ten purified proteins encoded by T4 phage: T4 DNA polymerase (gene 43 product), three polymerase accessory proteins which increase the affinity of polymerase for the growing chain, gene 32 single-stranded DNA binding protein, the genes 41 and 61 proteins which function together as a primase and as a DNA unwinding

enzyme (helicase), gene 59 protein which stimulates the primase-helicase, RNase H, and DNA ligase.

Mutations in T4 DNA polymerase affecting replication accuracy. T4 DNA polymerase has extensive regions with amino-acid sequence similarity to a large family of DNA polymerases including eukaryotic cellular and viral enzymes. In collaboration with M. Frey and S. Benkovic (Pennsylvania State University), they have shown that a single amino acid change (Asp-219 to Ala) within a highly conserved region decreases the 3'→5' exonuclease by at least 10^7 fold, with no detectible change in the polymerase activity. *In vivo*, the absence of the proofreading exonuclease in this polymerase mutant increases the frequency of mutations in the unlinked acriflavine resistance genes by an average of 760 fold.

Their recent studies of an "antimutator" (Ala-737 to Val) T4 DNA polymerase indicate that this mutation decreases the processivity of polymerase activity, and increases the processivity of the proofreading exonuclease, without changing the rate of hydrolysis of single-stranded DNA. These processivity changes are reversed by a second-site mutation (Leu-771 to Phe) that reduces the antimutator phenotype *in vivo*. These results suggest that the accuracy of the antimutator polymerase is improved by increasing the rate at which the growing strand of the duplex moves between the polymerase and exonuclease active sites on the enzyme.

Interactions between replication proteins. An important goal of these studies is to understand how interactions between different proteins in the multi-enzyme complex regulate synthesis of the leading and lagging strands. On the leading strand, T4 DNA polymerase follows behind the 41 protein helicase, whose activity is stimulated by the gene 59-DNA binding protein. A mutation in the polymerase, which decreases its processivity, prevents leading strand synthesis in the absence of 59 protein. Recent gel mobility studies demonstrate that the 41 and 59 proteins interact in the absence of DNA. DNA templates with photo-activatable cross-linkable residues in the fork ahead of the primer have been constructed, and are now being used to determine whether any of the accessory proteins are in front of polymerase, and to learn how and where the 61, 41, and 59 protein primase-helicase components assemble at the fork.

On lagging strand templates covered with 32 protein, the primase composed of the 61 and 41 proteins can synthesize primers only when either the polymerase accessory proteins or the 59 protein is available. RNA pentamers, made by the T4 primase in the absence of 32 protein, are elongated by polymerase and the accessory proteins only when the 61 primase protein is present. These findings are consistent with the possibility that the synthesis and elongation of the primers are coordinated by an interaction between the polymerase and primase complexes.

T4 RNaseH. The T4 phage RNaseH gene has been identified and cloned, and the purified enzyme shown to remove RNA primers *in vitro*. Recent work demonstrates that this T4 RNaseH is required to remove primers from the lagging strand *in vivo*. In a nonpermissive host defective in both *E. coli* RNaseHI and the 5' to 3' exonuclease of DNA polymerase I, a T4 mutant with a deletion in the phage RNaseH gene makes few viable progeny, and accumulates short DNA chains characteristic of unligated lagging strand fragments.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57501-17

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Function of DNA Virus Genomes in Animal Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Roland A. Owens	Senior Investigator	LMCB:NIDDK
Other:	Sandra Afione	Special Volunteer	LMCB:NIDDK
	Barrie Carter	Special Volunteer	LMCB:NIDDK
	Terence Flotte	Special Volunteer	LMCB:NIDDK
	Sirkka Kyostio	IRTA Fellow	LMCB:NIDDK
	Irving Miller	Biologist	LMCB:NIDDK
	Rikki Solow	Special Volunteer	LMCB:NIDDK
	Matthew Weitzman	Visiting Fellow	LMCB:NIDDK

COOPERATING UNITS *(if any)*

B. Safer, J. Chiorini, E. Urcelay (MH/NHLBI/NIH); R. Kotin (MH/NHBLI/NIH and Genetic Therapy Inc.); N. Chejanovsky (Bet-Dagan, Israel); P. Zeitlin, W. Guggino, D. Markakis (Johns Hopkins); M. Drumm (U. Michigan); D. Klessig (Rutgers).

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

Molecular Biology

INSTITUTE AND LOCATION

NIDDK:NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	-

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Adeno-associated virus type 2 (AAV) is a human parvovirus which usually requires adenovirus or herpesvirus as a helper to replicate and is not pathogenic. In the absence of helper virus, AAV DNA integrates into the host genome with a strong preference for a region of chromosome 19. The AAV *rep* gene open reading frame encodes four overlapping Rep proteins. Rep68 and Rep78 bind specifically to the AAV genome's inverted terminal repeats (ITRs), have enzymatic activities which are necessary for the resolution of AAV hairpin termini into linear DNA, and regulate AAV gene expression. We have expressed wild-type and mutant Rep proteins from recombinant baculoviruses in insect cells, a rabbit reticulocyte transcription-translation system, or the HIV-1 long terminal repeat promoter in human 293 cell transient transfections. Several parts of the Rep coding region have been identified as being necessary for the various Rep functions. In particular, we have identified a domain within the amino-terminal portion of the Rep78 and Rep68 proteins which can direct binding to the AAV ITRs. We have also demonstrated the repression of AAV RNA levels by Rep proteins. We have identified a binding site for Rep proteins near the chromosome 19 integration site. This implies a role for Rep proteins in directed integration. Further study of this phenomenon may lead to the development of AAV-based gene therapy vectors with site-specific integration. We have developed a gene delivery system based on (AAV) for potential gene therapy of cystic fibrosis (CF). A truncated version of the cystic fibrosis transmembrane conductance regulator cDNA flanked by the AAV ITRs, which is efficiently packaged into AAV capsids, corrected the CF chloride channel defect in a tissue culture cell line derived from lung cells of a cystic fibrosis patient. We have also discovered a cryptic promoter within the AAV terminal repeats. Using the ITR promoters, larger genes than previously thought possible can be packaged into AAV vectors.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57502-20

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Hormonal Regulation of Cell Growth and Differentiation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Takami Oka	Senior Investigator	LMCB:NIDDK
Other:	Robert Moore	IRTA Fellow	LMCB:NIDDK
	Seiji Nishikawa	Visiting Fellow	LMCB:NIDDK
	Norio Nonomura	Visiting Fellow	LMCB:NIDDK
	John W. Perry	Biologist (Technician)	LMCB:NIDDK

COOPERATING UNITS *(if any)*

Dr. Koichi Enomoto, Shimane Medical University, Japan
Dr. Sergio Lavandero, Department of Molecular Biology, University of Chile, Chile

LAB/BRANCH

Laboratory of Molecular & Cellular Biology

SECTION

Cell Growth and Differentiation Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Beta-Casein is the major milk protein synthesized by the mammary gland during lactation. Casein gene expression is regulated both positively and negatively by the complex interaction of peptide and steroid hormones. Beta-Casein gene expression can be induced by prolactin, glucocorticoid, and insulin, but is inhibited by progesterone. Since regulation of transcription involves specific interactions of the trans-acting factors with regulatory DNA elements, we examined nuclear factors that interact with the mouse Beta-casein promoter region during the development of the mammary gland by gel mobility shift assays using various probes that represent different portions of the promoter region. Our results indicated the presence of a mammary gland-specific binding protein with a mol wt. of 65 KD. This factor binds to a palindromic sequence, 5'-TGAT/ATCA-3', located at position -9/+4 and -363/-349. This factor has been detected in mammary tissue during pregnancy but not the virginal or lactational periods, and is termed pregnancy-specific mammary nuclear factor (PMF). The biological significance of PMF and its binding elements for casein gene transcription was examined by transfection experiments of Beta-casein promoter-CAT chimeric gene into mammary epithelial cells. Progesterone-mediated repression of transcription was derepressed by co-transfection with an oligo nucleotide containing the PMF binding site. Furthermore, mutation in PMF binding sites abolished the inhibitory effect of progesterone, whereas the mutant was still able to respond to the induction by lactogenic hormones. These results suggest that PMF is important for the repression of Beta-casein transcription by progesterone. To investigate whether the pregnancy-specific appearance of PMF is related to the increased serum level of progesterone, ovariectomy of pregnant mice and hormone replacement studies were performed. These studies showed that after ovariectomy the binding activity of PMF in the mammary gland was reduced to a very low level and that progesterone replacement specifically prevented the loss of PMF-binding activity. The changes in PMF activity were also inversely correlated with the alterations in casein gene expression in the tissue. The above results suggest that the repression of Beta-casein transcription by progesterone during pregnancy is mediated, at least in part, via PMF whose appearance is regulated by progesterone in the mammary gland.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57503-20

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Lysosomal Transport and Storage Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: Frank Tietze

COOPERATING UNITS *(if any)*

William A. Gahl, Human Genetics Branch, NICHD

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	-

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Work from several laboratories over the past 10-15 years has established that the monomeric end-products of lysosomal digestion, e.g. amino acids, monosaccharides, nucleosides and (probably) lipid components traverse the lysosomal membrane to the cytoplasm through the agency of specific carriers rather than by simple diffusion. The importance of these carriers to animal metabolism is indicated by the existence of two human disorders, viz., cystinosis and sialic acid storage disease, which owe their origin to inherited defects in the lysosomal transport systems for cystine and sialic acid,, respectively. To date, none of the aforementioned lysosomal transport systems has yet been purified or characterized by direct chemical approaches, nor has any of these carriers been cloned by genetic linkage studies or other recombinant DNA techniques. Although direct purification of membrane transportors is hampered by the lack of a suitable assay system, such difficulty has been overcome in the past by the use of reconstituted lipid membrane systems or proteolpiposomes, i.e., lipid vesicles incorporating the membrane proteins and which retain the original transport function. As a preliminary to employing this methodology to the purification of the lysosomal cystine carrier, we have prepared lysosomal membrane vesicles from rat liver and have investigated their characteristics with respect to cystine transport. Our studies have indicated that these vesicles possess cystine transport activity and may therefore serve as useful starting material in the preparation of functional preoteoliposomes for purification of the cystine carrier.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57504-06

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of HIV by AAV

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Roland A. Owens	Senior Staff Fellow	LMCB:NIDDK
Other:	Sirkka Kyostio	IRTA Fellow	LMCB:NIDDK
	Matthew Weitzman	Visiting Fellow	LMCB:NIDDK

COOPERATING UNITS *(if any)*

A. Rabson and B. Antoni (New Jersey Center for Advanced Biotechnology and Medicine)

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

Molecular Biology

INSTITUTE AND LOCATION

NIDDK:NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	-

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

(Note: This project will be joined with project Z01 DK 57501 for FY94). The wild-type Rep proteins of adeno-associated virus type 2 (AAV) have been demonstrated in tissue culture to inhibit the replication of HIV-1, the etiological agent of AIDS. Unfortunately, their potential as a therapeutic agent for AIDS is limited because they also appear to block cell division. Several groups have tried and failed to produce cell lines producing high levels of Rep proteins, although we have achieved high levels in transient expression assays in cells transfected with a plasmid expressing the *rep* gene from the long terminal repeat promoter of HIV-1 (HIV-LTR). Our objective is to create a mutant Rep protein which can block HIV-1 replication without blocking cell division. We have made a series of mutant *rep* genes expressed from the HIV-LTR. Our collaborators at the New Jersey Center for Advanced Biotechnology and Medicine have already tested several of these mutants for their ability to block HIV-1 replication. Plasmids containing the mutant *rep* genes are co-transfected with an infectious proviral clone of HIV-1 into human cells and the cells and culture supernatants are assayed for reverse transcriptase activity, HIV-1 RNA and for HIV-1 gag protein. We have identified several portions of the Rep proteins which are important for this inhibition. Several of these mutants inhibit HIV-1 better than the wild-type proteins. If we can identify a mutant Rep protein that blocks HIV-1 production without blocking cell division then we would have the basis for a novel treatment for AIDS. If Rep proteins truly block cell division then they may also be developed into a treatment for cancer. We will continue to produce and test mutant Rep proteins for HIV-inhibition. We will be concurrently attempting to produce cell lines making these mutant proteins. This will allow us to determine which parts of the Rep proteins are involved in the putative cell division block.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 DK 57800-02

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Initial Intracellular Events of Steroid Hormone Action

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	S.S. Simons, Jr.	Chief, Steroid Hormones Section	LMCB:NIDDK
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	Pradip K. Chakraborti	Visiting Associate	LMCB:NIDDK
	Clayton Collier	PRATT Fellow	LMCB:NIDDK
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	Justicia Opoku-Edusei	IRTA Fellow	LMCB:NIDDK
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	Daniele Szapary	Visiting Associate	LMCB:NIDDK

COOPERATING UNITS *(if any)*

D.P. Edwards (Univ. of Colorado, Denver), W.B. Pratt (Univ. of Michigan Medical School, Ann Arbor), K.R. Yamamato (UCSF, San Francisco), G. Schutz (German Cancer Research Center, Heidelberg, Germany)

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

Steroid Hormones Section

INSTITUTE AND LOCATION

NIDDK:NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
7.0	7.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is to define the initial, intracellular events of glucocorticoid hormone action and steroid hormone action in general. Such studies are of particular current relevance since steroid hormone receptors are arguably the best understood regulators of eukaryotic gene transcription. The first step of steroid binding to the intracellular receptor is followed by activation of the receptor-steroid complex to a DNA/nuclear-binding species that then associates with those nuclear acceptor sites involved in the regulation of transcription of selected genes in specific cells. We have found that the steroid binding domain of the glucocorticoid receptor not only binds steroid but also is sufficient for the association of a non-receptor protein, heat shock protein 90, which is required for steroid binding and whole cell localization of receptors. This result will greatly aid future molecular studies of the initial steps in steroid hormone action. The nuclear binding of activated complexes is required for the expression of steroid responses and is different for glucocorticoid and antiglucocorticoid steroids. We have found that this binding cannot be reproduced *in vitro* using methodologies that work for other macromolecules. This suggests that novel processes are involved in glucocorticoid receptor binding to nuclei. Efforts to define these new processes, and thus permit future *in vitro* studies of nuclear binding, should be facilitated by the use of aurintricarboxylic acid, which we found readily distinguishes between receptors bound to DNA and non-DNA acceptor sites. *In vivo* nuclear-bound activated receptor-steroid complexes often modulate the transcription of selected proteins in a tissue specific manner. Our finding of a new tissue selective element in the expression of a model glucocorticoid inducible gene represents a further advance towards identifying all of the components required for cell-free studies of tissue specific gene expression. Collectively, our current findings contribute to our long term goal of defining the mechanisms of steroid hormone action at a molecular level.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 DK 57801

PERIOD COVERED
October 1, 1992 through September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Enzymatic Mechanisms of DNA Replication: The Bacteriophage T4 System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Nancy G. Nossal	Chief, LMCB	LMCB:NIDDK
Other:	Lisa Hobbs	IRTA	LMCB:NIDDK
	Todd Capson	Senior Staff Fellow	LMCB:NIDDK

COOPERATING UNITS *(if any)*
Dr. Craig Hyde, LSBR, NIAMS
Dr. Stephen Benkovic, Dept. Chemistry, Pennsylvania State Univ., Univ. Park, PA

LAB/BRANCH
Laboratory of Molecular and Cellular Biology

SECTION
Section on Nucleic Acid Biochemistry

INSTITUTE AND LOCATION
NIDDK:NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.2	3.2	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

(This project was formerly ZO1 DK 24,260-26 LBP). We are continuing our study of the *E. Coli* bacteriophage T4 model system for duplex DNA replication in which efficient DNA replication *in vitro* is achieved with purified proteins encoded by T4 phage: T4 DNA polymerase (gene 43), gene 32 DNA helix-destabilizing protein, the gene 44/62 and gene 45 polymerase accessory proteins, the genes 41, 61, and 59 primase-helicase, RNase H, and DNA ligase. *Mutations in T4 DNA polymerase.* Our studies of an antimutator mutant in T4 DNA polymerase (A737V) indicate that the accuracy of the polymerase can be altered by changing the rate at which the growing strand of the duplex moves between the polymerase and exonuclease active sites on the enzyme. This single amino acid substitution decreases the processivity of the polymerase activity and increases the processivity of the proofreading exonuclease activity. These processivity changes are reversed by the compensating L771F mutation. *Interactions between replication proteins.* We are studying how interactions between proteins in the replication complex regulate synthesis on the leading and lagging strands. DNA templates with photo-activatable cross-linking residues in the fork ahead of the primer have been constructed, and are being used to determine whether any of the accessory proteins are in front of polymerase, and to learn how and where the 61, 41, and 59 protein primase-helicase components assemble at the fork. In studies of the mechanism by which the gene 59 protein stimulates the 41/61 protein primase-helicase, we have demonstrated a physical interaction between the 59 and 41 proteins in the absence of DNA. *T4 RNaseH.* In a nonpermissive (RNaseH defective) host, a T4 mutant with a deletion in the phage RNaseH gene makes few viable progeny, and accumulates short DNA chains characteristic of unligated lagging strand fragments. We are using purified T4 RNaseH to study how primer removal and gap filling are coordinated. *Structure of the T4 replication proteins.* We are collaborating with Craig Hyde, NIAMS, to try to crystallize these proteins.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 DK 57802

PERIOD COVERED
October 1, 1992 through September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Bacteriophage T4 Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Deborah M. Hinton	Research Chemist	LMCB:NIDDK
Other:	Mridula Sharma	Visiting Associate	LMCB:NIDDK
	Rosyln March-Amegadzie	Senior Staff Fellow	LMCB:NIDDK

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Molecular and Cellular Biology

SECTION
Section on Nucleic Acid Biochemistry

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	-

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

(This project was formerly Z01 DK 23,750-05 LBP). During infection, different classes of bacteriophage T4 genes are transcribed by the host RNA polymerase, which is modified by the phage at middle and late times. Transcription from middle promoters also requires the T4 MotA protein. We have been investigating the mechanism of MotA at the T4 middle promoter PuvsX. Like other *motA*-dependent promoters, PuvsX contains a match to the sequence (t/a) (t/a) TGCTT (t/c) A ('MotA box'), centered at -30 relative to the start of transcription. In addition, other possible MotA boxes are found in PuvsX, centered at -35, -51, -70, and -87. Our DNase I footprint analyses indicate that MotA protects PuvsX in the region from -25 to -59. This includes the MotA box at -30, but also the farther upstream sites centered at -35 and -51. *In vitro* transcription of a template lacking the MotA box at -51 indicates that while this site is not required for activation, it contributes to MotA action under less favorable transcription conditions. The first 8 amino acids (aa) of MotA are replaced with 11 different aa in the MotA mutant protein Mot21. Although Mot21 binds PuvsX similarly to wild type MotA, it does not activate transcription. In addition, C-terminal fragments of MotA, starting at aa 102 or aa 105, also bind PuvsX DNA. These results identify the C-terminal half of the protein as the DNA binding domain and suggest that the N-terminal half may be involved in protein-protein interactions. The T4 *segA* gene encodes a Mg++-dependent endonuclease, which is related by sequence to endonucleases present in group I introns. Our characterization of the SegA cleavage activity indicates that although SegA prefers to cleave at certain sites, as the concentration of protein or incubation time is increased, any DNA is digested. Linear cleavage products are ligated by T4 DNA ligase, and SegA activity is stimulated by the addition of ATP. Thus, SegA exhibits a hierarchy of site preference, and the apparent specificity of SegA is governed by the level of the protein, the length of incubation, and the presence of any factors that can stimulate or reverse the reaction.

1992-3

ANNUAL REPORT OF THE LABORATORY OF ANALYTICAL CHEMISTRY NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The primary mission of the Laboratory of Analytical Chemistry (LAC) focuses on the provision of analytical services for investigators in NIDDK and elsewhere at NIH and the development of improved analytical methods. Many of the analytical problems brought to LAC for solution involve only application of relatively routine methods, while others are of such complexity that collaborative research efforts are needed.

The instrumental analysis capabilities of LAC are concentrated mainly on nuclear magnetic resonance (NMR), mass spectrometry (MS) and infrared spectroscopy (IR). No new instruments were acquired this year, but continuing upgrades have augmented the Laboratory's spectral apparatus. In addition to instrumental analysis, the Laboratory has processed nearly 100 samples for over 4000 microanalytical analyses.

Developments in Mass Spectrometry

Mass spectral analysis is often crucial to the identification of products that have been synthesized or separated from mixtures. Nearly 6000 samples have been analyzed in LAC mass spectrometry labs, most of which can be characterized as "routine" analyses, but an increasing number have required extensive research for interpretation of the data. For example, Whittaker has collaborated with Kovac and Kovacik (LMC, NIDDK) to develop methods for sequence analysis of heterooloigosaccharides, while Pannell has collaborated with NCI investigators to solve the structures of several natural products which show promising anti-HIV activities.

The program begun last year to extend MS capabilities of LAC to the area of biopolymers has continued with the development of methods for determining modifications in proteins that arise from natural biological processes or from labelling experiments. For example, Pannell, et al. have located the phosphorylation site of prothymosin α and are investigating the active site of aldose reductase.

Applications of NMR

Over 800 "routine" NMR spectra have been provided to investigators in NIDDK and other Institutes. Increasingly, however, investigators have taken advantage of the "hands-on" NMR spectrometer capability in LAC to obtain several thousand of their own spectra, with guidance and assistance as needed from LAC staff. During the last year the GEMINI-300 NMR spectrometer was used for this purpose a total of over 5000 hours, which represents about 60 percent of the available time.

Among the more complex collaborative research problems under investigation during the last year were studies of colchinoid inhibitors of tubulin polymerization, covalent nucleoside adducts from diol epoxides of carcinogenic polycyclic aromatic hydrocarbons, DNA duplexes which are

chemically modified with highly carcinogenic diol epoxide metabolites of benzo[a]pyrene, and acid catalyzed rearrangement products of deoxoartemisinins, antimalarial drugs.
Other NMR research centers on a study of the mechanism of formation of isomeric nucleosides. A number of N-7 isomers of ribo- and deoxyribo-nucleosides have been prepared and used in the NMR studies. This work is near completion and is in the preparation for publication.

Organizational Changes

The Biophysical Histology Section was abolished, and a new Structural Mass Spectrometry Unit was established under Dr. Lewis Pannell.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z-01 DK58000-48 LAC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analytical Service and Methodology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Edwin D. Becker	Acting Chief, LAC	LAC/NIDDK
Others:	Herman J. C. Yeh	Research Chemist	LAC/NIDDK
	Lewis K. Pannell	Visiting Scientist	LAC/NIDDK
	Noel Whittaker	Chemist	LAC/NIDDK
	Wesley White	Biologist	LAC/NIDDK

COOPERATING UNITS *(if any)*

Laboratory of Bioorganic Chemistry, NIDDK; Laboratory of Medicinal CHemistry, NIDDK; Laboratory of Biophysical CHemistry, NHLBI

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Instrumentation Section

INSTITUTE AND LOCATION

NIH/NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.3	2.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to NIDDK scientists, other NIH laboratories and, to a limited extent, personnel of other government agencies. Instrumental analyses include nuclear magnetic resonance of liquids and solutions; infrared spectroscopy of solids, liquids, and samples introduced by gas chromatograghy; mass spectrometry (with samples introduced by solids probe, gas and liquid chromtography and capillary zone electrophoresis with ionization by electron impact, chemical ionization, electrospray, thermospray, thermabeam, and fast atom bombardment.

Requests for analyses are obtained from many laboratories and Institutes at NIH, as well as some outside organizations. The laboratory in this way acts as an NIH resource and it is supported, in part, by its collaborators in terms of staffing and equipment where appropriate. Some major instruments are jointly funded and shared with other Institutes to avoid costly duplication of facilities.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 58003-19

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Development of Methods and Materials for the Study of Medical Problems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C.M. Foltz Research Chemist LAC/NIDDK

Others:

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION NIDDK,NIH, Bethesda, Maryland

TOTAL MAN-YEARS: 0	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Project Terminated

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-DK 58004-26 LAC

PERIOD COVERED
October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Professional Practices of Biomedical Scientist

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Feder	Medical Officer (Research)	LAC/NIDDK
Others:	W.W. Stewart	Research Physicist	LAC/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH Laboratory of Analytical Chemistry

SECTION Biophysical Histology

INSTITUTE AND LOCATION
NIH/NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 0	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Project Terminated

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-DK-58007-08 LAC

PERIOD COVERED
October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
PHYSOSTIGIMINE AND ANALOGS

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi — Visiting Scientist — LAC/NIDDK

Others:

COOPERATING UNITS (if any)
Drs. Nigel Greig and S. I. Rapoport, Laboratory of Neuroscience, NIA, NIH; Dr. Q. S. Yu, Institute of Organic Chemistry, Shanghai, China; J. L. Flippen-Anderson, Laboratory of the Structure of Matter, Naval Research Laboratory, Washington, D.C.

LAB/BRANCH Laboratory of Stuctural Biology

SECTION Natural Products Section

INSTITUTE AND LOCATION
NIH/NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 0	PROFESSIONAL: 0	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Project Terminated

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-DK-58011-16 LAC

PERIOD COVERED
October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
STRUCTURE-ACTIVITY RELATIONSHIPS OF COLCHICINOIDS BASED ON TUBULIN BINDING

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi Visiting Scientist LAC/NIDDK

Others:

COOPERATING UNITS (if any)
Dr. E. Hamel, LMP, National Cancer Institute, NIH; Dr. P. Gros, Department of Biochemistry, McGill University, Montreal, Canada; Dr. V. Simanek, Institute of Medicine, Palacky University, Olomouc, Czechoslovakia

LAB/BRANCH Laboratory of Structural Biology

SECTION Natural Products Section

INSTITUTE AND LOCATION
NIH/NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 0	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Project Terminated

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-839

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-DK58012-01 LAC

PERIOD COVERED
October 1, 1992 to September 30, 1993

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Application of NMR Spectroscopy in Chemical and Biochemical Analysis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Herman J. C. Yeh	Research Chemist	LAC/NIDDK
Other:	Guiying Li	Visiting Fellow	ROB/NCI

COOPERATING UNITS *(if any)*
Laboratory of Bioorganic Chemistry, NIDDK; Laboratory of Chemical Physics, NIDDK; Laboratory of Molecular Pharmacology, NCI; Radiation Oncology Branch, NCI; Research Division, Hoffman-LaRoche, Inc.

LAB/BRANCH
Laboratory of Analytical Chemistry

SECTION
Instrumentation Section

INSTITUTE AND LOCATION
NIH/NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Various NMR techniques have been used to solve the solution structures of a number of molecules. These include colchinoids, acid catalyzed rearrangement products of deoxoartemisinins, and covalent nucleoside adducts from diol epoxides of polycyclic aromatic hydrocarbons.

We have analyzed 500 MHz NMR NOE spectra of a DNA duplex modified at the adenosine residue with a highly carcinogenic diol epoxide metabolite of benzo[a]pyrene. These NOE data in conjunction with computer spectral simulation and structure modeling will be used to determine the solution structure of the modified DNA duplex.

In an attempt to gain insight into the mechanism of the formation of the less receptive isomeric nucleosides by purine nucleoside phosphorylase, a number of N-7 isomers of ribo- and deoxyribo-nucleosides have been synthesized and their reaction products in the presence of the enzyme have been monitored by NMR. This work is near completion.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK58013-01 LAC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mass Spectrometry of Drugs, Natural Products, Proteins and Oligonucleotides.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute

L.K. Pannell	Visiting Scientist	LAC, NIDDK
D. Uyakul	Visiting Fellow	LAC, NIDDK
P. Lecchi	Visiting Fellow	LAC, NIDDK
Q-l. Pu	Special Volunteer	LAC, NIDDK
A. McKinney	IRTA Fellow	LAC, NIDDK
T. Carroll	Stay-in-school	LAC, NIDDK

COOPERATING UNITS (if any)

Daly, Spande and Garraffo, LBC, NIDDK; Fales and Sheeley, LBC, NHLBI; Rodgers, LCB, NIDDK; Munro and Blunt, U. Canterbury, New Zealand; Berger, DCBDC, NCI; Rogawski and Yamaguchi, ERB, NINCDS; Gustafson, Beutler and Boyd, NCI, FCRC;

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Structural Mass Spectrometry Unit, Instrumentation Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Specialized mass spectrometry analyses are provided to the laboratory and to other collaborating units. The emphasis of this work is split between identification of trace organic compounds isolated from biological sources and the mass spectrometry of biopolymers (e.g. peptides, proteins and oligonuceotides). A very close working relationship and collaboration is maintained with the Laboratory of Biophysical Chemistry, NHLBI, especially with the research into, and use of techniques for, mass spectrometry of macromolecules. The electrospray source on the JEOL SX102 instrument was enhanced by the addition of a camera which allows easy optimization of the spray during analyses. The capillary zone electrophoresis instrument continues to receive heavy usage from this laboratory and others in the Institute. This has also been interfaced to the electrospray source where its separation efficiency far surpasses that of traditional HPLC methods. As part of the macromolecule collaboration with LBC, NHLBI, routine use is made of a mass spectrometer especially set up for sequencing of protein digests. In addtion, use is made of a Laser Desorption mass spectrometer in NCI and in LBC, NHLBI (Dr. Fales) for the analysis of proteins and protein digests as part of our expansion into biopolymer mass spectrometry. In the small molecule area, collaborative interest has continued in the identification of biologically active natural products, especially those of interest in AIDS research and treatment; several anticonvulsant plasma drug level studies have been completed in collaboration with NINCDS. Samples analyzed derive from many facilities and researchers.

ANNUAL REPORT OF THE LABORATORY OF NEUROSCIENCE

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

Studies on the benzodiazepine/GABA receptor chloride channel complex

The benzodiazepine/GABA receptor chloride channel complex ("supramolecular complex") is an oligomeric group of proteins containing recognition sites for many psychopharmacological agents including benzodiazepines, ß-carbolines, and barbiturates. The proteins comprising this supramolecular complex act in concert to regulate the activity of chloride channels that are controlled ("gated") by gamma-aminobutyric acid (GABA), the principal inhibitory neurotransmitter of the vertebrate central nervous system. Studies are in progress to characterize the molecular aspects of this system, its physiological functions, and possible role in disease.

Recent molecular biological studies indicated the supramolecular complex required at least three distinct but related classes of proteins (termed alpha, beta and gamma) to form a fully functional drug and ligand-gated chloride channel. The establishment of a stable cell line containing cDNAs encoding only two of these classes of proteins (alpha, gamma) that expresses "Type I" benzodiazepine receptors indistinguishable from the native form suggests that the $GABA_A$ receptor complex may also be constituted in heterodimer forms. Nonetheless, neither the number nor arrangement of subunits required to constitute this ligand-gated ion channel are known. Current studies are underway to resolve this issue as well as examine the potential for regulation by (e.g.) drugs in this stably transfected cell line.

The discovery of several high affinity ligands for the "diazepam-insensitive" isoform of benzodiazepine receptor should enable us to design novel agents with selectivity for these sites. These studies should permit us to perform molecular modelling studies of this isoform as well as to elucidate potential pharmacological and physiological functions. This may be of particular significance since Ro 15-4513 (the prototypic ligand for diazepam-insensitive benzodiazepine receptors) can antagonize many of the effects of ethanol in both electrophysiological and biochemical measures and has amethystic properties *in vivo*.

Previous studies from this laboratory suggest that the $GABA_A$ receptor complex may mediate the pharmacological effects of inhalation agents. The demonstration than an inhalation agent can produce anesthesia in a stereospecific manner *in vivo* is consistent with a protein rather than lipid target of anesthesia, and permits further study of the locus of action of these agents.

Studies on glycine and glutamate coupled cation channels

We previously demonstrated that functional antagonists at the N-methyl-D-aspartate (NMDA) receptor complex exhibit antidepressant actions in animal models. The high affinity glycine partial agonist, 1-aminocyclopropanecarboxylic acid (ACPC) was as efficacious as the prototypic antidepressant, imipramine, in these

measures. This is of particular importance since ACPC is orally active, has a long duration of action, and does not appear to produce the adverse behavioral effects associated with competitive NMDA antagonists or use-dependent channel blockers. The development of a simple HPLC analytical method to measure this material in biological fluids indicates that both plasma and brain concentrations of this compound are consistent with pharmacological actions in behavioral measures. Moreover, the demonstration that chronic administration of both ACPC and MK-801 produce a downregulation of ß-adrenoceptors comparable to imipramine indicates that glutamatergic pathways may be a common pathway of antidepressant drug action. This hypothesis is currently under investigation. The finding that chronic treatment with ACPC results in a desensitization of the NMDA receptor complex led to the demonstration that this regimen effectively reduced the mortality and improved the neurological status of animals subjected to severe forebrain ischemia. The feasibility of applying this regimen to other neuropathologies associated with excessive activation of the NMDA receptor complex is currently under investigation.

The snail toxin Conantokin-G was shown to act as an NMDA antagonist through a specific, noncompetitive inhibition of polyamine responses. However, this peptide appears to act at a previously undescribed locus on the NMDA receptor complex. Studies are currently underway to modify this peptide in order to determine the minimum size and sequence required to produce this action. This and related peptides should be valuable tools in examining the physiological importance of polyamines and their recognition sites in the operation of this ligand-gated cation channel.

Studies on neural-immune interactions

We previously demonstrated that the presence of the LP-BM5 virus mixture in the central nervous system of mice inoculated with this virus mixture as neonates. This virus mixture produces a profound immunosuppression and has been proposed as a murine model of AIDS (MAIDS). The demonstration of cognitive (memory and learning) deficits in these animals using a modified Morris water maze indicates these mice may be a useful non-primate model to study the neuropsychiatric consequences of AIDS. This model may be of particular value since cognitive deficits were evinced prior to the appearance of gross motor or other neurological deficits. Current studies are underway to determine whether drugs that prevent or reduce viral invasion of the central nervous system will affect the cognitive deficits produced by this virus mixture.

Using a simple and reliable method for dual-color analysis of heterogenesis cell populations, it was previously shown that inhibition of calcium influx into splenocytes may be an early event in immunosuppression produced by both physiological (stress) and pharmacological (opiates) means. The finding that pharmacologically relevant concentrations of ethanol and related alcohols effect a similar reduction in mitogen-induced increases in intracellular free calcium is consistent with the notion that this phenomenon may be common to immunosuppression produced by diverse factors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK,58501-07

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Receptors for Neurotransmitters & Drugs in Brain & Peripheral Tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: P. Skolnick, Chief, LN; A.S. Basile, Pharmacolgist; L. Fossom, Staff Fellow; B. Hawrris, Guest Researcher; R. Layer, Guest Researcher; E. Mody, Special Volunteer; R. Muhkerjee, Special Volunteer; G. Nowak, Visiting Fellow; I.A. Paul, Staff Fellow; Y. Sei, Visiting Associate; G. Wong, Guest Researcher; L.-M. Zhou, Visiting Fellow; Y. Li, Visiting Associate; P. Popik, Visiting Fellow; J.H. Ha, Special Volunteer; R. Trullas, Special Volunteer.

COOPERATING UNITS (if any)

C. Yurdaydin, DDB, NIDDK; K.C. Rice, LMC, NIDDK; B. deCosta; LMC, NIDDK; K. Jacobsen, LBC, NIDDK; cooperating units cont. pg 2

LAB/BRANCH

Laboratory of Neuroscience

SECTION

Section on Neurobiology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
12.5	12.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

High affinity, sterospecific recognition sites (receptors) for neurotransmitter, neuromodulators,and many clinically useful drugs have been identified in both peripheral tissues and the central nervous system. The interaction of a neurotransmitter, neuromodulator or drug with a specific recognition site initiates a series of events (for example, the opening of an ion channel or activation of an enzymes) resulting in either a physiological response (in the case of a neurotransmitter or neuromodulator) or a pharmacological effect (in the case of a drug). Furthermore, the presence of recognition sites for synthetic substances indicates that endogenous substances may also be present which can mimic (or antagonize) the effects of exogenously applied (synthetic) compounds. Studies are in progress to characterize "recognition-effector" systems with special emphasis on ligand (transmitter)-gated ion effector systems, to isolate and identify novel endogenous ligands of physiological and pathophysiological importance, to develop appropriate model systems to examine these phenomena, and to relate recognition-effector systems to both physiological and pathophysiological processes.

ANNUAL REPORT OF THE MOLECULAR PATHOPHYSIOLOGY BRANCH
National Institute of Diabetes and Digestive and Kidney Diseases

The general goals of the Branch are to investigate normal and abnormal cell function at the molecular level with emphasis on transmembrane signalling by hormones, neurotransmitters, growth factors, and other first messengers acting at the cell surface. Approaches used range from molecular biologic techniques to clinical investigation in an effort to define the pathogenesis of diseases characterized by abnormal signal transduction.

Guanine nucleotide binding proteins (G-proteins) as receptor-effector couplers

A family of G-proteins functions in transmembrane signalling as receptor-effector couplers. G-proteins couple to a diverse array of receptors including those for polypeptide hormones, monoamine neurotransmitters, photons of light, chemical odorants, chemotactic factors, and certain growth factors. Effector functions regulated by G-proteins include cAMP formation, cGMP degradation, phosphoinositide breakdown, and several types of ion channel. Major areas of interest concerning G-proteins include: 1)definition of the diversity within this gene family; tissue and subcellular distribution; regulation of gene expression. 2)definition of domains on individual G-protein subunits involved in association of the subunits, attachment to cell membranes, interaction with receptor and effector domains, and possible interactions with other regulatory proteins. 3)definition of the degree and mechanism of specificity for individual G-proteins in coupling to both receptors and effectors. 4)definition of quantitative and qualitative alterations in G-proteins that result in altered signal transduction. Significant recent progress has been made in each of these areas:

1) Molecular basis for subunit association and membrane attachment of GTP-binding proteins- G-proteins are heterotrimers; alpha subunits reversibly associate with a beta/gamma complex. The holoprotein is associated with the cytoplasmic side of the plasma membrane, but the basis for membrane attachment and the domains responsible for subunit association have not been defined. By metabolic labeling of transfected cells and immunoprecipitation of expressed alpha subunits, we can show that Gi but not Gs-alpha subunits undergo a specific fatty acid acylation (myristoylation). The latter is a cotranslational modification, dependent upon protein synthesis, and irreversible. A myristoylation defective Gi alpha subunit is expressed solely in the soluble fraction.This highlights the crtitical importance of this modification for G alpha membrane attachment, but begs the question of the mechanism of attachment of Gs- and Gq-alpha which do not undergo myristoylation. We now find that G-alpha subunits, including Gs-alpha, undergo palmitoylation. In contrast to myristoylation, palmitoylation is a post-translational modification that is reversible and independent of protein synthesis. Site directed mutagenesis of cysteine-3 in Gs-alpha blocks palmitoylation. This is the likely site for this modification. We are currently studying the functional significance of the modification. Interestingly, we have found that palmitoylation of

Gs-alpha is apparently stimulated in intact cells by activation of a receptor (beta-adrenergic) coupled to Gs. This suggests a model in which activation of Gs-alpha leads to dissociation from the beta/gamma dimer, and palmitoylation of Gs-alpha occurs perhaps to help preserve membrane association. The structural basis for Gs-alpha membrane targeting (as distinct from attachment) remains unclear. Extensive mutagenesis studies including amino and carboxy terminal as well as double deletions indicate that neither the extreme amino- nor carboxy-termini are critical for Gs-alpha localization to the particulate fraction of transfected cos cells. This may reflect the role of multiple domains in Gs-alpha membrane attachment. Regions of Gs-alpha shown to be critical for effector interaction are by themselves incapable of targeting the protein to the cell membrane. [Drs.Jones, Detgarjev, and Spiegel]

2) Studies of the structure and function of the βγ dimer:

a) Characterization of γ subunit structure and mapping of functional domains- The shift of β subunit immunoreactivity to the cytosol upon cotransfection with nonprenylated γ provides an assay of βγ complex formation. The domain(s) of γ critical for selectivity in βγ complex formation are being mapped by exploiting the ability of nonprenylated γ2, but not γ1, to shift β2 to the cytosol upon cotransfection. A series of γ1/γ2 chimeras have been constructed to localize the region of γ critical for β selectivity in heterodimer formation. In addition a series of N- and C-terminally truncated γ mutants are being used to determine the minimum structure necessary for coexpression with β. [Drs. Lee, Garritsen and Simonds]

b) Functional effects of γ mutation on βγ targeting and function in stable transfectants- C6 glioma cells were stably transfected with the nonisoprenylated γ_2 mutant and immunoblot analysis revealed that a portion of endogenous β subunits were shifted to the cytosol. This demonstrated that the exogenous mutant γ was capable of assembling with and diverting at least a portion of the endogenous β pool to the cytosol. Preliminary functional studies of β-adrenergic stimulated cAMP formation in stable transfectants reveal a blunted response to agonist in mutant γ-bearing but not control cells, consistent with a dominant inhibitory phenotype. [Drs. Simonds and Spiegel, with Dr. Collins]

c) Mutagenesis of the β subunit. The amino terminus of the β subunit is predicted by computer algorithms to form an α-helix and to favor formation of a coiled-coil interaction. Unlike wild-type β, an amino-terminal β deletant which lacks the coiled-coil region fails to shift to the cytosol when coexpressed with the nonisoprenylated γ_2 mutant. Such results led to the recognition of a coiled-coil domain in the γ

subunit and generation of a molecular model of βγ interaction based on a coiled-coil interaction in which a core hydrophobic interaction is reinforced by flanking ionic bonds between the two α-helices. [Drs. Garritsen and Simonds, in collaboration with Dr. Van Galen]

d) Effector modulation by the βγ complex:

Transient cotransfection of COS cells with phospholipase C-β2 and G-protein β1 and γ2 cDNAs increases inositol phosphate formation relative to vector-transfected cells. Preliminary results confirm that the nonisoprenylated γ_2 mutant fails to demonstrate this effect. Such a paradigm may allow screening of β and γ mutants for effector function.

A 28-amino acid domain comprising the βγ binding site has been identified in the carboxyl-terminus of the β-adrenergic receptor kinase (βARK). This domain is nearly coincident with a 29-amino acid region predicted by computer algorithm to favor coiled-coil formation. This has led to the hypothesis that βARK-βγ interaction may involve the reversible formation of a three-stranded coiled coil structure. [Drs. Manji, Garritsen and Simonds, in collaboration with Drs. de Blasi and Lupas]

3) Altered G-proteins as a cause of altered signal transduction- As critical intermediates in the signal transduction pathway, quantitative or qualitative alterations in G-proteins could have a major impact on the signalling process. We have generated mutant alpha subunits for several G proteins that should lead to either constitutive activation or dominant inhibition of G protein function. The mutant alpha subunits have been initially characterized after transient expression in cos cells. Mutant Gi2-alpha cDNAs have been stably transfected into NIH 3T3 cells. The constitutively active mutant causes increased cell proliferation, while the dominant inhibitory mutant slows growth.Comparable mutants of Gi1 also stimulate cell proliferation when stably transfected in NIH3T3 cells but those of Gi3-alpha do not. In the latter case, immunolocalization studies indicate that Gi3 may be preferentially targeted to the golgi rather than plasma membrane. This raises interesting questions regarding differential distribution and function of specific G protein subtypes. Overexpression of various G-alpha subunits in stable cell lines was found to increase expression of beta subunits. This provides the first evidence for coordinate regulation of G protein subunit expression. The mechanism for such regulation requires further study. [Drs. Hermouet, Merendino, and Spiegel]

4) Specificity of G protein receptor-effector coupling- We have made specific peptide antibodies against G protein alpha subunits. Antisera raised against the carboxy-termini of alpha subunits are capable of uncoupling receptor from G protein in native membranes, and can be used to define the specificity of coupling. In collaboration with a group at UCSD, affinity purified antibodies have been microinjected into living cells to permit direct demonstration of the role of specific G protein alpha subunits in key signal transduction responses. The Gi2-specific

antibody, for example, blocked mitogenesis in response to serum factors in swiss 3T3 cells, whereas the Gq antibody (specific for a novel class of pertussis toxin-insensitive G proteins linked to phospholipase C stimulation) blocks increases in intracellular calcium and mitogenesis in response to bradykinin. [Drs. Shenker, Goldsmith, and Spiegel, in collaboration with Drs. Unson and Feramisco]

Pseudohypoparathyroidism (PHP)

PHP is a genetic disorder in which resistance to parathyroid hormone (PTH) may be associated with somatic abnormalities collectively termed Albright's hereditary osteodystrophy (AHO). We have previously shown that subjects with this form of PHP are resistant to multiple hormones that act by stimulating cAMP formation, that an approximate 50% reduction in activity of the G-protein (Gs) that couples receptors to stimulation of adenylyl cyclase is present in all tissues from affected subjects, and that subjects with PHP show reduction in steady state mRNA for the Gs-alpha subunit. We have now succeeded in defining the genetic abnormality responsible for Gs deficiency. Using the polymerase chain reaction to amplify genomic fragments encompassing each exon of the Gs-alpha gene, and comparing such fragments from normal and affected subjects on denaturing gradient gel electrophoresis, we were able to identify fragments with abnormal mobility. By direct DNA sequencing such fragments contained mutations that would explain reduction in mRNA in affected subjects. This work indicates that mutations in a G protein gene can lead to clinically significant disease. [Drs. Weinstein, Friedman and Spiegel, in collaboration with Dr. Gejman]

McCune-Albright syndrome (MAS)

MAS is a non-genetic disorder in which affected subjects show a variety of seemingly unrelated abnormalities including polyostotic fibrous dysplasia, pigmented skin lesions (cafe-au-lait spots), and autonomous hyperfunction of various endocrine organs including gonads, anterior pituitary, thyroid, and adrenal cortex. The endocrine abnormalities lead to precocious puberty, gigantism, hyperthyroidism, and hypercortisolism. The cause of this sporadic disorder has been completely enigmatic, but speculations have centered on a defect in signal transduction leading to endocrine hyperfunction. The distribution of skin lesions has also suggested the possibility of a somatic mutation acquired early in embryogenesis and affecting only a subset of cells (mosaicism). Since a G protein mutation could plausibly explain the endocrine manifestations, we searched for and found mutations of the Gs-alpha gene that lead to constitutive activation of the Gs protein. These mutations were found in a mosaic distribution; notably, mutant gene was undetectable in normal-appearing portions of endocrine glands, but was present at heterozygous levels in neoplastic portions of endocrine tissue. Mutant Gs-alpha was also detected in dysplastic bone lesions. Occurrence of mutant Gs-alpha in organs such as heart and liver suggest a possible role in "non-classical" manifestations, including sudden death. Our studies suggest that MAS is

caused by a somatic mutation in the Gs-alpha gene occurring early in development and found in a mosaic distribution. [Drs. Weinstein, Shenker and Spiegel, in collaboration with Dr. Gejman]
McCune-Albright syndrome (MAS)

Nephrogenic diabetes insipidus (NDI)

NDI is an inherited X-linked disorder in which affected subjects are resistant to the actions of vasopressin (AVP) on renal medullary cells responsible for water concentration. Clinical manifestations include severe polydipsia and polyuria, and resultant severe dehydration can lead to cerebral swelling and death. Treatment with a potent AVP analog (DDAVP), useful in other forms of DI, is ineffective in NDI because of end-organ resistance to the hormone. The renal actions of AVP are mediated through a V2 type receptor linked via the Gs protein to stimulation of the 2nd messenger cAMP. In theory, the inherited gene defect could be located anywhere along the signal transduction path, but indirect evidence suggested a likely receptor defect. The recent cloning of a human V2 receptor permitted chromosomal localization studies which showed that the receptor is localized to Xq28, the site of the gene defect as determined by family linkage studies. This strongly suggested but did not prove that a receptor gene mutation is the underlying defect in NDI. We have obtained genomic DNA samples on multiple families with NDI, and in three families thus far have identified mutations predicted to disrupt formation of a normal V2 receptor. These findings have important implications for our understanding of the pathogenesis of NDI and of normal V2 receptor structure and function, for identification of affected subjects and carriers, and eventually for gene therapy of the disease. [Drs. Merendino and Spiegel, in collaboration with Drs. Lolait and Brownstein, NIMH].

Molecular biologic studies on the cause of parathyroid neoplasia

Parathyroid tumors (benign adenomas, hyperplasia, and carcinoma) are presumptively due to acquired (and in some cases such as multiple endocrine neoplasia type I {MEN I} to inherited) abnormalities at the gene level. We are studying the molecular basis for parathyroid neoplasia by searching for mutations, rearrangements and/or deletions in genomic DNA from parathyroid tumors. We have found rearrangement of the parathyroid hormone gene in only 1 of 43 parathyroid adenomas, but this gene abnormality may be pathogenetically relevant. In contrast, point mutations in ras oncogenes were not found in any parathyroid tumors. In both "hyperplastic" glands from subjects with MEN I and in sporadic adenomas loss of heterozygosity for loci on chromosome 11q13 was found. The data show that tumors in MEN I are monoclonal, and that a locus on 11q13 may encode a tumor "suppressor" gene. By mapping deletions at 11q13 in parathyroid tumors, we are attempting to identify the MEN I gene.

Honors and Awards:

Lee Weinstein (Senior Staff Fellow) gave a lecture at the Annual Meeting of the Endocrine Society.

Teresa Jones (Medical Staff Fellow) was invited to speak at the Monod Conference in France on post-translational lipid modifications of proteins.

William Simonds (Medical Staff Fellow) was invited to speak at the Monod Conference in France on GTP-binding proteins and signal transduction.

Andrew Shenker (Senior Staff Fellow) was invited to speak at a Serono Symposium in Italy on GTPases.

Allen Spiegel (Branch Chief) was invited to lecture at National and International Meetings including the 4th Joint Meeting of the American and European Pediatric Endocrine Societies, the Gordon Conference on Bones and Teeth, the National Meeting of the Japanese Endocrine Society, and the 2nd Gerald Aurbach Memorial Lecture of the American Society for Bone and Mineral Research.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-DK- 59000-06 MPB

PERIOD COVERED
October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecular biologic studies on the cause of parathyroid neoplasia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D. Chief, MPB, NIDDK

Others: E. Friedman, M.D. Senior Staff Fellow, MPB, NIDDK

COOPERATING UNITS (if any)
S. Marx, M.D., Chief, Mineral Metaholism Section, MDB, NIDDK

LAB/BRANCH: Molecular Pathophysiology Branch

SECTION:

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD

TOTAL STAFF YEARS 0.5	PROFESSIONAL: 0.5	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Primary hyperparathyroidism (HPT), a common endocrine disorder that can cause significant morbidity, may be due to benign neoplasia of a single parathyroid gland (adenoma) or multiple parathyroid glands (hyperplasia), and rarely, to malignant neoplasia of a parathyroid gland (carcinoma). The etiology of parathyroid neoplasia has not been defined. As with other forms of neoplasia, parathyroid tumors are presumably due to inherited (germ-line mutation) and/or acquired (somatic mutation) defects in specific genes. Etiologic genetic defects could include inappropriate expression of transforming "oncogenes" and/or loss of expression of tumor "suppressor" genes. The availability of surgically resected parathyroid tumors from patients with sporadic and hereditary forms of disease allows us to search for tumor-specific genetic abnormalities that may be involved in development of parathyroid neoplasia.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-DK 59001-28 MPB

PERIOD COVERED
October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Guanine nucleotide binding proteins as receptor-effector couplers

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A. Spiegel, M.D.,	Chief, MPB, NIDDK	
Others:	T. Jones, M.D.	Senior Clinical Inv., MPB, NIDDK	A. Shenker, M.D.,
	P. Goldsmith, Ph.D.	Res. Biol., MPB, NIDDK	S. Hermouet, M.D.,
	Y.-S. Juhnn, M.D.	Visiting Fellow, MPB, NIDDK	N. Thambi, Ph.D.
	M. Degtjarev	Visiting Fellow, MPB, NIDDK	

COOPERATING UNITS (if any)
C. Unson, Rockefeller Univ., N.Y.
J. Feramisco, UCSD, LA Jolla, CA

LAB/BRANCH: Molecular Pathophysiology Branch

SECTION:

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD

TOTAL STAFF YEARS 8	PROFESSIONAL: 6	OTHER: 2

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A family of guanine nucleotide binding proteins (G-proteins) functions in transmembrane signalling as receptor-effector couplers. G-proteins couple to a diverse array of receptors including those for hormones, neurotransmitters, light, odorants, and certain growth factors. Effector functions regulated (positively and, in some instances, negatively) by G-proteins include cAMP formation, phosphoinositide breakdown, potassium and calcium channels, and cGMP degradation. We have used a variety of techniques to study the expression, distribution, regulation, structure and function of G-proteins. Our studies highlight the diversity within the G-protein family. Using peptide specific antibodies, we have defined the specificity of G-proteins in coupling to receptors and effectors. We have defined distinct post-translational lipid modifications necessary for membrane attachment of G protein alpha and beta/gamma subunits. We have created mutations in alpha subunits that cause constitutive activation, and transfected these into cells to define phenotypic effects on cellular function. These studies provide the basis for understanding the role of G-proteins in normal signal transduction and for elucidating possible defects in G-protein structure or function as the basis for abnormal signal transduction.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 59002-28 MPB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on pseudohypoparathyroidism and related disorders

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. Weinstein, M.D. Senior Staff Fellow, NIDDK, MPB

Others: A. Spiegel, M.D. Chief, MPB, NIDDK

S.-H. Yu, Ph.D. Visiting Associate, MPB, NIDDK

P. DeMazancourt, Ph.D. Visiting Fellow, MPB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH Molecular Pathophysiology Branch

SECTION

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS 1.0	PROFESSIONAL: 0.5	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [X] (b) Human tissues [ ] (c) Neither

[X] (a1) Minors

[ ] (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism" (PHP). Patients with this disorder show characteristic constitutional features (Albright's hereditary osteodystrophy - AHO) and do not respond to exogenous parathyroid hormone (PTH). In PHP, UcAMP (urinary cyclic AMP) does not increase normally in response to PTH administration. This indicates that there is a defective hormone receptor-adenylate cyclase complex in this disorder. We have shown that many patients with PHP+AHO (PHP Ia) show an approximately 50% reduction in activity of Gs (the stimulatory guanine nucleotide binding protein associated with adenylate cyclase) in membranes from multiple tissues. Gs deficiency presumably accounts for resistance to multiple hormones in such patients. Using cloned human cDNA probes for the alpha subunit of Gs, we have shown that steady state mRNA levels from fibroblasts of subjects with PHP Ia are reduced by approximately 50% compared with normals. We have now succeeded in defining the genetic abnormality responsible for Gs deficiency. Using the polymerase chain reaction, denaturing gradient gel electrophoresis and direct sequencing, we were able to identify mutations that would explain reduction in mRNA in affected subjects. Other patients express renal resistance to PTH but no features of AHO or resistance to other hormones. Previous work in this laboratory showed a selective resistance of cAMP generation to PTH in fibroblasts from these patients, suggesting a potential defect in the PTH receptor.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-DK 59003-03 MPB

PERIOD COVERED
October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Studies on McCune-Albright Syndrome

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D. Chief, MPB, NIDDK

Others: L. Weinstein, M.D. Senior Staff Fellow, MPB, NIDDK
A. Shenker, M.D, Ph, D. Senior Research Inv., MPB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH Molecular Pathophysiology Branch

SECTION

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD

TOTAL STAFF YEARS 1	PROFESSIONAL: 1	OTHER: 0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

McCune-Albright syndrome (MAS) is a non-inherited disorder in which affected subjects show a variety of seemingly unrelated abnormalities including the classic triad of polyostotic fibrous dysplasia, pigmented skin lesions (cafe-au-lait spots), and autonomous hyperfunction of various endocrine organs including gonads, anterior pituitary, thyroid, and adrenal cortex. The endocrine abnormalities lead to precocious puberty, gigantism, hyperthyroidism, and hypercortisolism. The cause of this sporadic disorder had been completely enigmatic, with speculations centered on a defect in signal transduction leading to endocrine hyperfunction. The distribution of skin lesions has also suggested the possibility of a somatic mutation acquired early in embryogenesis and affecting only a subset of cells (mosaicism). Since a G protein mutation could plausibly explain the endocrine manifestations, we searched for and found mutations of the Gs-alpha gene that lead to constitutive activation of the Gs protein. These mutations were found in a mosaic distribution; notably, mutant gene was undetectable in normal-appearing portions of endocrine glands, but was present at heterozygous levels in neoplastic portions of endocrine tissue. Mutant Gs-alpha was also detected in dysplastic bone lesions. Occurrence of mutant Gs-alpha in organs such as heart and liver suggest a possible role in "non-classical" manifestations, including sudden death.Our studies suggest that MAS is caused by a somatic mutation in the Gs-alpha gene occurring early in development and found in a mosaic distribution.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-DK 59004-02 MPB

PERIOD COVERED
October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Guanine nucleotide binding protein beta-gamma dimers: structure and function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	William Simonds, M.D.,	Senior Clinical Investigator	MPB, NIDDK
Others:	A. Garritsen, Ph.D.,	Visiting Fellow,	MPB, NIDDK
	C. Lee, Ph.D.	Visiting Fellow,	MPB, NIDDK
	H. Manji	Senior Staff Fellow,	ETC, NIHM

COOPERATING UNITS (if any)
C. Unson, Rockefeller University; P.J. M. Van Galen, LBC, NIDDK
A. DeBlasi, Consorzio Mano Negis Sad, Italy
R. Collins, MPB, NIDDK; A. Lupas, Max Planck Instit. for Biochem., Germany

LAB/BRANCH: Molecular Pathophysiology Branch

SECTION:

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD

TOTAL STAFF YEARS	PROFESSIONAL	OTHER
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The guanine-nucleotide binding regulatory proteins (G-proteins) are $\alpha 1\beta 1\gamma 1$ heterotrimers which function as transmembrane signal transducers by coupling receptors for extracellular stimuli to intracellular effectors (enzymes, ion channels). G-proteins constitute a diverse family distinguished by specific receptor and effector interactions which in turn are determined by the structure of the three constituent subunits. The α subunit binds guanine nucleotides and has a well established role in effector modulation. The β and γ subunits are tightly associated as a $\beta\gamma$ complex, comprising a single functional entity which, like the α subunit, is absolutely required for G-protein interaction with receptor. An effector modulatory role for the $\beta\gamma$ complex is becoming increasingly apparent in several systems. The present research emphasizes the role of the $\beta\gamma$ complex in G-protein-mediated signal transduction. We have used subunit specific peptide antibodies to probe regions of the $\beta\gamma$ complex important for functional interaction with the α subunit and to monitor expression of recombinant subunits. Site-directed mutagenesis has been used to study the assembly, processing and effector function of the $\beta\gamma$ complex in both transient and stable transfected cell systems. These studies may elucidate the contribution of the $\beta\gamma$ subunit complex to the receptor and effector selectivity characteristic of G-proteins and to the adaptive responses pursuant to agonist stimulation.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-DK 59005-02 MPB

PERIOD COVERED
October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Studies on nephrogenic diabetes insipidus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D. — Chief, MPB, NIDDK

Others: J. Merendino, M.D. — Clinical Associate, MPB, NIDDK
N. Thambi, Ph.D. — IRTA, MPB, NIDDK
P. Goldsmith, Ph.D. — Research Biologist, MPB, NIDDK

COOPERATING UNITS (if any)
M. Brownstein, S. Lolait, Laboratory of Cell Biology NIMH

LAB/BRANCH: Molecular Pathophysiology Branch

SECTION:

INSTITUTE AND LOCATION: NIDDK, NIH Bethesda, MD

TOTAL STAFF YEARS	PROFESSIONAL	OTHER
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [x] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Nephrogenic diabetes insipidus (NDI) is an inherited X-linked disorder in which affected subjects are resistant to the actions of vasopressin (AVP) on renal medullary cells responsible for water concentration. Clinical manifestations include severe polydipsia and polyuria, and resultant severe dehydration can lead to cerebral swelling and death. Treatment with a potent AVP analog (DDAVP), useful in other forms of DI, is ineffective in NDI because of end-organ resistance to the hormone. The renal actions of AVP are mediated through a V2 type receptor linked via the Gs protein to stimulation of the 2nd messenger cAMP. In theory, the inherited gene defect could be located anywhere along the signal transduction path, but indirect evidence suggested a likely receptor defect. The recent cloning of a human V2 receptor permitted chromosomal localization studies which showed that the receptor is localized to Xq28, the site of the gene defect as determined by family linkage studies. This strongly suggested but did not prove that a receptor gene mutation is the underlying defect in NDI. We have obtained genomic DNA samples on multiple families with NDI, and in three families thus far have identified mutations predicted to disrupt formation of a normal V2 receptor. These findings have important implications for our understanding of the pathogenesis of NDI and of normal V2 receptor structure and function, for identification of affected subjects and carriers, and eventually for gene therapy of the disease

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ANNUAL REPORT OF THE LABORATORY OF MEDICINAL CHEMISTRY
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The major research direction of the Laboratory is the elucidation of the structure and function of neurotransmitter systems in the mammalian central nervous system (CNS) and the molecular mechanism of action of CNS active drugs. Also under investigation are the detailed interaction, on a molecular level, between antibodies and antigens, peripheral signaling systems and the mechanisms through which the immune and other peripheral systems are influenced by the CNS in normal and disease states, and the exploration of novel approaches to drugs to control cellular proliferation and virus growth. Organic/medicinal chemistry is the foundation of the multidisciplinary approach utilized in these studies which requires synthesis of novel agonists, antagonists, imaging agents, affinity ligands and other drugs for particular applications.

Drug Design and Synthesis Section

Present work in the Drug Design and Synthesis Section of this Laboratory is concerned with rational design and the synthesis of new, highly selective ligands for drug receptors, using all of the contemporary tools of medicinal chemistry, including computer assisted molecular modeling. Areas now under intense investigation include: (1) central opioid receptor subtypes and peripheral opioid receptors, (2) binding sites on components of the immune system which resemble central opioid receptor subtypes, (3) the mechanism of cocaine and narcotic tolerance and dependence (4) phencyclidine (PCP) recognition sites, (5) sigma, cannabinoid (marijuana) and central and peripheral benzodiazepine receptors and (6) development of new ligands for positron emission tomography (PET) and single photon emission computed tomography (SPECT) imaging of drug receptors in the CNS of living animals and conscious humans. The multidisciplinary nature of this program requires extensive collaboration with other groups from within and outside of NIH for the purpose of discernment of the structure and function of these receptors and to ensure the practical utility of the discovered ligands provided to biological and biochemical researchers. This Section is involved in collaborative work with, among others, researchers at the University of Alabama, the University of Arizona, the University of Michigan Medical School, the Medical College of Virginia, The University of Illinois Medical School in Peoria, the Naval Research Laboratory, the Walter Reed Army Institute of Research, the National Institute of Mental Health and the National Institute on Drug Abuse (ADAMHA), the Nuclear Medicine Department of the Warren Grant Magnuson Clinical Center of NIH, the National Heart, Lung and Blood Institute of NIH, the National Institute of Neurological Disorders and Stroke of NIH, G. D. Searle and Co., Neurogen Corp., and the Laboratory of Neuroscience of NIDDK.

Receptor Biochemistry and Pharmacology Unit of the Drug Design and Synthesis Section

This Unit is engaged in the elucidation of the structure and function of sigma receptors. Sigma receptors are membrane-bound proteins found in the brain, spinal cord, and some peripheral tissues. These sites are distinct, both in pharmacological profile and tissue distribution, from any known neurotransmitter or hormone receptor and exist in at least two subtypes. They are of interest because of their ability to bind members of several important classes of psychoactive drugs. These include typical antipsychotic drugs such as haloperidol, psychotomimetics such as phencyclidine, and the antitussive/ anticonvulsant agent dextromethorphan. Though the exact role of sigma receptors in synaptic transmission is still largely unknown, sigma sites have been implicated in a number of biochemical, physiological, and behavioral processes. First recognized by their high affinity for typical antipsychotic drugs, sigma sites have been implicated in not only the positive effects of these agents, but also in the untoward motor side effects resulting from acute and chronic treatment. Sigma sites have also been implicated in regulation of: normal motor function, smooth muscle contraction, dopamine synthesis and release, neuronal firing rate, muscarinic phosphoinositide response, NMDA receptor function, intracellular calcium concentration, and drug metabolism. The ability of sigma agents to protect neurons from damage during ischemic insult to the brain or spinal cord has led to investigations of the role of sigma receptors in neuronal growth and degeneration. Functions in the digestive system have also been demonstrated. The localization of sigma sites in nervous as well as non-nervous tissue suggests a variety of other functions of these receptors and points to the vast clinical potential of sigma agonists and/or antagonists. We have interest in all aspects regarding the biochemistry and molecular pharmacology of this receptor system, and we are utilizing preparations

of brain, various peripheral tissues, as well as neuronal and non-neuronal cells in culture as model systems.

Behavioral Pharmacology Unit of the Drug Design and Synthesis Section

The primary mission of the Behavioral Pharmacology Unit is to assess pharmacological agents which have the potential for modifying or antagonizing the effects of drugs of abuse such as cocaine, using drug self-administration paradigms in the rhesus monkey. The examined drugs are designed and synthesized in the Drug Design and Synthesis Section or are obtained from other sources. The laboratory has developed several additional capabilities to facilitate the understanding of relevant end points of drugs of abuse related to abuse liability. For example, neuroendocrine and immune function, as well as other behavioral end points, can be evaluated initially in rodent models. The ability to PET scan monkeys that have acquired self-administration behavior is being explored. We have established collaborative efforts with the local neuroscience community to provide additional support, primarily using behavioral genetics as a tool. In addition, we have established behavioral assays for additional ligand-behavior interactions which may be relevant to unique effects of drugs of abuse. Lastly, we have continued the development of techniques which identify adverse properties of drugs and other agents which are considered undesirable (behavioral toxicology).

Biomedical Chemistry Section

In the Biomedical Chemistry Section, LMC, interferon-induced enzyme activities such as the 2-5A synthetase, the 2',5'-phosphodiesterase, and the 2-5A-dependent ribonuclease are studied with the goal of understanding their role in the action of interferon, the induction of interferon by dsRNA, and the control of cell growth and differentiation. Analogues of a mediator of interferon action are synthesized in order to define the relationship between oligonucleotide structure and binding to and activation of the 2-5A-dependent endonuclease. The eventual goal is to understand the biological role of the 2-5A system and to explore the potential of exploitation of this system in chemotherapy. Finally, a number of new approaches to pharmacologically active nucleoside analogues are pursued.

Section on Carbohydrates

This Section works on the interaction of (complex) carbohydrate determinants with monoclonal antibodies (MAbs). The elucidation of this interaction - in great molecular detail - is important since it pertains to all ligand-protein interactions. Thus, drug-receptor, effector-receptor as well as viral-receptor interactions may be clarified. We are executing:

1. Physico-chemical studies on antibody/antigen systems.
2. The synthesis of ligands for affinity studies.
3. The manipulation of immunoglobulin genes to produce genes expressing altered antibodies.
4. The study of immunodeterminants of bacteria causing significant diseases on a global scale, so as to evaluate procedures for vaccine development.

We are continuing to determine the specific interaction between microbial polysaccharides such as dextran and a number of monoclonal antibodies, and have prepared ligands to probe the fundamental nature of the antibody-antigen association.

We have prepared additional complex fragments of the capsular polysaccharide of *Shigella dysenteriae* type 1 by sophisticated syntheses. These include deoxy derivatives of the determinant Studies are continuing on the binding area of a monoclonal antibodies towards this disease-causing micro-organism.

The variable region of the heavy (V_H) and the light (V_L) chains have been cloned and sequenced, and they have been incorporated in the bacterial expression vector pSW1, connected by a linker coding for a fifteen amino acid peptide. The expressed protein is a covalently linked sF_V. It occurs in inclusion bodies. Attempts to solubilize it in an active form have not yet been successful. This Section is involved in collaborative research with scientists at Columbia University, in NICHD, NIH, and in Czechoslovakia.

The following summary describes selected advances made in the three Sections of the Laboratory of Medicinal Chemistry during 1992-1993.

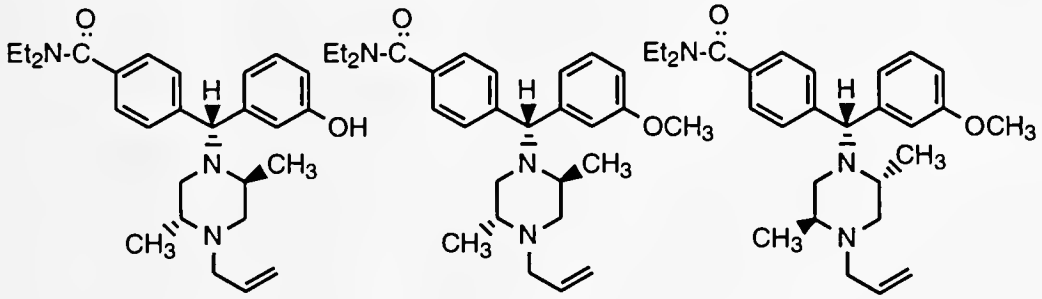
DRUG DESIGN AND SYNTHESIS SECTION

Opioid Receptors

Identity and Function of Delta Opioid Receptor Subtypes. Recent pharmacological data strongly support the hypothesis of delta (δ) receptor subtypes as mediators of both supraspinal and spinal antinociception (δ_1 and δ_2 receptors). In vitro ligand binding data which are fully supportive of the in vivo data are still lacking. A previous study indicated that [^{3}H][D-Ala$_2$,D-Leu$_5$]enkephalin labels two binding sites in membranes depleted of binding sites by pretreatment with the site directed acylating agent, 2-(*p*-ethoxybenzyl)1-diethylaminoethyl-5-isothiocyanatobenzimidazole.HCl (BIT). The main goal of the present study was to develop a ligand-selectivity profile of the two δncx binding sites. The data indicated that naltrindole and oxymorphindole were relatively selective for site 1 (20-fold). [D-Ser$_2$,Thr$_6$]enkephalin and deltorphin-II were only 2.7-fold and 2.2-fold selective for site 1. [D-Pen$_2$,D-Pen$_5$]enkephalin and deltorphin-I were 80-fold and 38-fold selective for site 2. 3-Iodo-Tyr-D-Ala-Gly-Phe-D-Leu was 52-fold selective for site 1. Morphine had moderate affinity for site 1 (Ki=16 nM), and was about 11-fold selective for site 1. Thus of the 10 drugs studied, only DPDPE and DELT-I were selective for site 2. Viewed collectively with other data, it is likely that the δ1 receptor and the δncx binding site are synonymous.

Research into the functional role of the opioid δ receptor has intensified with the recent in vivo identification of δ receptor subtypes, termed δ_1 and δ_2. which mediate antinociception in the mouse. A variety of data also support the hypothesis of an opioid receptor complex composed of distinct, yet interacting mu (μ), δ, and perhaps kappa (κ) binding sites. This model postulates two classes of δ binding sites: a δ binding site not associated with the opioid receptor complex, termed the δ_{ncx} site, and a δ site associated with the receptor complex, termed the δ_{cx} site. A major purpose of this study was to clarify the relationship between the δ_{ncx} binding sites and the δ_1 and δ_2 receptors. Mouse brain membranes were depleted of μ sites by pretreatment with the site directed acylating agent, BIT, and the δ_{ncx} binding sites were labeled with [^{3}H][D-Ala$_2$,D-Leu$_5$]enkephalin. Binding surface analysis readily resolved two binding sites ($\delta_{ncx\text{-}1}$ and $\delta_{ncx\text{-}2}$) in the absence and presence of 100 mM NaCl. Control experiments with guanine nucleotides and the ligand-selectivity analysis indicated that the two sites were not two states of a single receptor. Pretreatment of membranes with DALCE, but not [Cys$_4$]deltorphin, decreased [^{3}H][D-Ala$_2$,D-Leu$_5$]enkephalin and [^{3}H][D-Ser$_2$,Thr$_6$]enkephalin binding. Ligand-selectivity analysis of the two binding sites suggested that neither δ_{ncx} binding site had the characteristics expected of the δ_2 receptor, and that the $\delta_{ncx\text{-}1}$ site, but not the $\delta_{ncx\text{-}2}$ site, was synonymous with the δ_1 receptor. Moreover, our finding that the racemic non-peptide δ agonist, BW373U86, had high affinity at and selectivity for the $\delta_{ncx\text{-}2}$ site, suggests that this site may be a novel δ receptor which mediates some of the effects of BW373U86.

Identification of Nonpeptide Ligands with a Remarkable 2000-fold Selectivity for Delta vs. Mu Opioid Receptors. The synthesis of potent non-peptide agonists and antagonists and affinity labels with high selectivity for δ opioid receptor subtypes would provide valuable research tools for gaining further insight into the structure and function of the δ opioid receptor system. A novel nonpeptidic δ-opioid receptor agonist, ((±)-BW373U86), was recently reported. This potent racemic compound was shown to have a high degree of selectivity for δ opioid receptors in both *in vitro* receptor binding studies and in *ex vivo* functional assays.



(±)-BW373U86 SNC80 SNC83

Since it is well established that drug enantiomers can show distinctly different, and in some cases opposite, pharmacological effects, we thought that it would be of interest to study the optically pure enantiomers of BW373U86. We have developed a practical synthesis of the enantiomers of (±)-BW373U86 and its diastereoisomer, which retains the *trans* dimethyl piperazine moiety, and have synthesized related compounds as well. Our synthetic approach involves assembly of the molecule from two components: a) the chiral N-allyl-*trans*-2,5-dimethyl-1,4-piperazine and b) the appropriate benzhydryl chloride. The absolute configuration of an intermediate was determined by single-crystal X-ray diffraction. We evaluated the affinity of these isomers and their immediate precursors for δ and μ receptors through displacement studies. Opioid activity was studied in isolated mouse vas deferens (MVD) and guinea pig ileum (GPI).

The phenolic enantiomers of BW373U86 and their benzylic epimers show high affinity for δ and μ receptors, but the methyl ethers of two of these compounds (SNC80 and SNC83) show about 2000 fold δ selectivity in binding assays. Displacement studies of the methyl ether isomers of BW373U86, immediate precursors in our synthetic route, revealed that methylation of the phenolic group virtually eliminates the affinity of these compounds for μ receptors. Our results in the MVD and in GPI suggest that SNC80 is a highly selective and potent nonpeptidic δ agonist which will be of value for further elucidation of δ receptor function. Additional work with SNC80 as a template is being carried out to provide highly selective affinity labels, imaging agents and other research tools.

Drug Testing Program of the College On Problems Of Drug Dependence. The College (formerly, Committee) on Problems of Drug Dependence (CPDD) and its Drug Evaluation Committee (DEC), are involved with drug abuse research and the determination of the physical dependence potential and abuse liability of analgesics, stimulants and depressants. Data provided by the DEC are useful for scheduling decisions by U.S. federal agencies and the World Health Organization.

Immunoregulatory Opioids

Mu Opioid Receptor Subtype Mediation of Natural Killer Cell Activity in Vivo. The CNS opioid receptor subtypes responsible for opioid effects on natural killer (NK) cytotoxicity were examined by microinjecting opioid receptor-selective agonists into the lateral ventricles (i.c.v.) of Fischer 344N rats. Dose ranges of 20-200 nM (-)-(1*S*,2*S*)-U50,488 and 60-200 nM [D-Pen$_{2,5}$]-enkephalin (DPDPE), selective for κ and δ receptors, respectively, did not affect NK cytotoxic activity; however, the lowest DPDPE dose, 20 nM, increased NK cytotoxicity. In contrast, 60-200 nM of the selective μ agonist [D-Ala-, N-Me-Phe$_4$, Gly-ol]-enkephalin (DAGO) reduced NK activity. This reduction was blocked by pretreatment with naltrexone (5 mg/kg, i.p.). These findings indicate that opioid-induced immunosuppression is mediated primarily through central μ receptors. DAGO (60 nM) had no effect on plasma corticosterone or ACTH levels, suggesting that central μ binding does not reduce NK activity through activation of the hypothalamic-pituitary-adrenal-axis.

We have accumulated data indicating a differential effect mediated by δ-class selective opioid compounds on Ig production by mitogen-stimulated splenic and Peyer's patch lymphocytes. These results may be due to a selective distribution of opioid receptors on immunocytes which populate these organs and/or unique populations of lymphocytes residing in the gut associated lymphoid tissue compared to the spleen. The observations that the reduction in Ig production by the mitogen-stimulated lymphoid populations occurs in the presence of opioids is consistent with previous observations showing α-endorphin could suppress Ig production to sheep red blood cells and ovalbumin. Interestingly, the data indicate the δ-selective antagonist, naltrindole is active on lipopolysaccharide-stimulated splenic lymphocytes while the δ-selective agonist oxymorphindole is not. The role reversal observed in these cells is rather unique since it is not seen in Peyer's patch lymphocytes nor centrally within the brain. However, since oxymorphindole and naltrindole antagonize one another, they appear to operate through similar pathways, e.g., the δ-class opioid receptor. With the observations that cells of the immune system respond to opioid substances through classical ligand-receptor interactions indicates the presence of these receptors on the immunocyte membranes. In fact, recently an opioid receptor binding site has been purified from immunocytes. The expression of these receptors on lymphocytes and their function in vivo in immune homeostasis is currently being investigated.

A sigma receptor binding site on lymphocytes has previously been shown. However, no functional studies were described following receptor activation. In the present study, we employed [^{3}H](+)-pentazocine to identify and characterize the putative sigma receptors on lymphocytes. [^{3}H]pentazocine was found to specifically label high and low affinity sigma-type binding sites on lymphocytes. The binding was saturable with the high affinity site having a Kd value of 0.7 ± 0.24 nM and the lower affinity site having a Kd value of 1.8 ± 0.6 nM. Likewise, saturable high (Kd = 21 ± 9 pM) and low Kd = 415 ± 40 pM) affinity sites for [^{3}H]pentazocine were found on thymocytes as well. In competition studies, the rank order of potency for competing drugs is (+)-pentazocine = N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2(1-pyrrolidinyl)ethylamine > 1*R*,2*S*-(+)-*cis*-N-(2-(3,4-dichlorophenyl)ethyl]-2 -(1-pyrrolidinyl)cyclohexylamine > (-)-pentazocine ≥ phenazocine > (±) -*trans*-3,4-dichloro-N-methyl-N-[2-(l-pyrrolidinyl)cyclohexyl]benzene acetamide methane sulphonate = phencyclidine ≥ haloperidol = 1,3-di-(*o*-toly)-guanidine. Neither naloxone nor β-endorphin inhibited [^{3}H]pentazocine binding out to a concentration of 10 μM. (+)-pentazocine suppresses: (i) concanavalin A-stimulated lymphocyte proliferation, (ii) concanavalin A-elicited polyclonal IgM production, and (iii) $^{45}Ca^{2+}$ uptake by nonactivated and concanavalin A-activated lymphocytes. (-)-pentazocine augments concanavalin A-elicited polyclonal IgM production, induces interleukin-4 production by lipopolysaccharide-treated lymphocytes, enhances $^{45}Ca^{2+}$ uptake by nonstimulated lymphocytes, and enhances lymphocyte production of cAMP. These results indicate a stereoselective receptor for (+)-pentazocine which is coupled to biological processes of lymphocytes.

Studies Towards the Development of a Cocaine Antagonist

The need for an effective antagonist of cocaine and other psychomotor stimulants has assumed greater urgency as the widespread abuse of these drugs continues. The reinforcing effects of these compounds are thought to be due primarily to their ability to cause release and/or block the uptake of dopamine into nerve terminals associated with the reward pathways of the mesolimbic dopaminergic system . Because a positive correlation has been demonstrated between the rewarding properties of these drugs and their affinity for a cocaine receptor on the dopamine transporter labeled using [^{3}H]mazindol, it was hypothesized that compounds which prevent the binding of stimulants to this site might prove to be effective as cocaine antagonists.

Identification of Fourfit as a Selective Affinity Label for the Dopamine Transport Complex. Fourphit, a phencyclidine derivative containing an isothiocyanate substitution at the 4-position of the piperidine ring, inhibits the binding of the radiolabeled psychomotor stimulant, [^{3}H]methylphenidate, to sites on the dopamine transport complex in membranes prepared from the crude synaptosomal fraction of rat striatal tissue with an IC50 of 7.1 μM. The inhibition caused by fourphit is irreversible and is associated with a decrease in the Bmax, but not the Kd of [^{3}H]methylphenidate binding. Pretreatment with saturating concentrations of unlabeled methylphenidate effected a modest (but statistically significant) protection of the stimulant binding site from inactivation by fourphit, indicating that the acylating phencyclidine derivative may act directly at this site. Preincubation with fourphit rather than vehicle did not alter the dissociation rate of [^{3}H]methylphenidate when measured in the presence of excess amfonelic acid, nor was any difference detected in the off-rate of [^{3}H]methylphenidate when excess fourphit was substituted for excess unlabeled methylphenidate as the displacing agent. This lack of effect on the dissociation kinetics of [^{3}H]methylphenidate provides further evidence that fourphit does not act allosterically at the methylphenidate binding site. Unlike metaphit (an isomer of fourphit containing the isothiocyanate moiety at the *meta* position of the aromatic ring), fourphit can discriminate between the methylphenidate binding site and the phencyclidine binding site associated with the N-methyl-D-aspartate receptor: Metaphit irreversibly inactivates both binding sites, whereas fourphit binds reversibly to the phencyclidine binding site. The data suggest that fourphit may be useful as a relatively selective affinity label for the site on the dopamine transport complex recognized by methylphenidate and other psychomotor stimulants.

Heterogeneity of Dopamine Transport Complex Binding Sites for Dopamine Uptake Inhibitors. RTI-55 (3β-[4'-iodophenyl]tropan-2β-carboxylic acid methyl ester) is a cocaine congener with high affinity

for the dopamine transporter (Kd < 1 nM). The present study characterized [^{125}I]RTI-55 binding to membranes prepared from rat caudate nucleus. Using the method of binding surface analysis, two binding sites were resolved: a low capacity site (Bmax=846 fmol/mg protein) and a high capacity binding site (Bmax=11900 fmol/mg protein). The Ki values of selected drugs at the two sites were (Ki-low capacity site, Ki-high capacity site): RT1-55 (0.18 nM, 0.78 nM), GBR12935 (327 nM, 0.84 nM), mazindol (542 nM, 38 nM), 2β-carbomethoxy-3β-(4-fluorophenyl)tropane (51.2 nM, 126 nM) and cocaine (203 nM, 678 nM). Serotonergic (fluoxetine) and noradrenergic (nisoxetine) uptake blockers had low affinity for both binding sites. 6-Hydroxydopamine lesions of the caudate decreased the Bmax of both binding sites in the ipsilateral caudate by different degrees, indicating that the two binding sites are physically separable. Dopamine uptake inhibitors which do not produce cocaine-like effects in humans (mazindol, nomifensine, benztropine, bupropion and GBR12909), termed type 2 agents, were all greater than 14-fold selective for the high capacity binding site, whereas cocaine and its congeners were less than 3.5-fold selective for the high capacity binding site. Viewed collectively, these data provide evidence for a low capacity binding site which has high affinity for cocaine and its congeners, but low affinity for type 2 DA uptake inhibitors. Further studies with agents recently synthesized in our program will determine their selectivity and possible utility as agents for the treatment and prevention of cocaine abuse.

Phencyclidine Recognition Sites

We have studied the action of phencyclidine (PCP, 1-(1-phenylcyclohexyl)piperidine)-like ligands on glutamate receptors of the N-methyl-D-aspartate (NMDA) type. Phencyclidine binding sites have been implicated as allosteric sites which interact with glutamate receptors of the NMDA type. Some phencyclidine (PCP)-like compounds have recently been reported to exert a robust protective effect against neuronal degeneration in ischemia models; evidence suggests they act as antagonists against the depolarizing action of NMDA in animal brain. Other sites for interaction with PCP-like ligands in the CNS have also been found, including the dopamine uptake complex.

Synthesis and Binding Properties of MK-801-Isothiocyanates; (+)-3-Isothiocyanato-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine Hydrochloride: A New, Potent and Selective Electrophilic Affinity Ligand for the NMDA Receptor-Coupled Phencyclidine Binding Site. Three new site-directed irreversible (wash-resistant) ligands for the high-affinity phencyclidine (PCP) binding site associated with the N-methyl-D-aspartate (NMDA) receptor were synthesized and their binding characteristics were studied. (+)-3- And (+)-2-isothiocyanato-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine hydrochloride ((+)-**8a,b**.HCl) were prepared in four steps from the corresponding nitro derivatives (+)-4**a,b**, which were obtained by nitration of (+)-**3** (MK-801). In the same way the optical antipode (-)-**8a**.HCl was synthesized from (-)-**3**. At a concentration of 100 nM, the 3-isothiocyanate derivative (+)-**8a** irreversibly labeled approximately 50% of the (+)-[^{3}H]-**3** binding sites, compared to 20 mM needed for its optical antipode (-)-**8a** and the 2-isothiocyanate (+)-**8b**. The apparent K_i values for reversible inhibition of (+)-[^{3}H]-**3** binding by (+)- and (-)-**8a** and (+)-**8b** were 37, 838 and 843 nM, respectively. In contrast, metaphit (**1b**) and etoxadrol-*meta*-isothiocyanate (**2b**), two previously reported irreversible ligands for the PCP binding site, label about 50% of the (+)-[^{3}H]-**3** binding sites at 100 μM and 250 nM, respectively, with apparent K_i values for reversible inhibition of 535 nM and 94 nM. Compound (+)-**8a** is also a selective affinity ligand, displaying little or no irreversible in vitro affinity at 100 mM for opioid, benzodiazepine, muscarinic and dopamine receptors. At a 25 μM concentration, (+)-**8a** caused an irreversible 52% reduction of binding to σ_1-receptors. Compound (+)-**8a** is the most potent known electrophilic affinity label for the PCP binding site. Its potency and selectivity should enable it to be a valuable tool for the elucidation of the structure and function of the NMDA receptor-associated PCP binding site in the mammalian central nervous system.

Phencyclidine Binds to Blood Platelets With High Affinity and Specifically Inhibits Their Activation by Adrenaline. The ion channel probe phencyclidine [1-(1-phenylcyclohexyl)piperidine; PCP] selectively inhibited aggregation, secretion and ultrastructural changes in platelets induced by adrenaline, but did not affect activation induced by other common platelet agonists such as α-thrombin, ADP, collagen or ionophore A23187. [^{3}H]PCP bound to platelets with high affinity (Kd

134±33 nM; 3600±1020 sites/platelet), as did the thienyl analogue [^{3}H]TCP {1-[1-(2-thienyl)cyclohexyl]piperidine}. PCP binding to platelets was increased 3-4-fold in N-methylglucamine buffer in the absence of Na^+ ions. Binding was unaffected by haloperidol and was only weakly inhibited (EC_{50} 10-20 mM), without significant stereoselectivity by the two sets of stereoselective ligands, dexoxadrol/levoxadrol and (+)MK801/(-)MK801. Binding of PCP was not competed for by adrenaline or yohimbine. Only the high-affinity binding of [^{3}H]PCP to platelets was blocked by prior treatment of the platelets with the covalent affinity probe metaphit, and these platelets no longer aggregated in response to adrenaline although they responded normally to α-thrombin, ADP and collagen. These results suggest that platelets contain high affinity receptors for PCP that can modulate adrenaline-induced platelet activation.

Structure-Activity Studies on (+)-3-Isothiocyanato-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*] cyclohepten-5,10-imine. In contrast to (+)-3- isothiocyanato-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*] cyclohepten-5,10-imine hydrochloride [(+)-**8a**], both the (-)-3-isothiocyanate (-)-**8a** and the 2-isothiocyanate (+)-**8b** show only low affinity for the PCP site. The fact that the optical antipode of (+)-**8**a is not very effective is in accordance with the probable asymmetric nature of this receptor site. Although (+)-**3** (MK-801) and its optical antipode only differ in activity by a factor of 7, increasing the asymmetry by adding a substituent increases the in vitro differences between the enantiomers. The inactivity of the (+)-2-isothiocyanate derivative appeared to be puzzling. However, it can be presumed that the nucleophilic moiety on the receptor, with which the isothiocyanate must react, has to be in close proximity to make covalent binding to the receptor possible. There is no *a priori* certainty that (+)-**8b** will covalently bind to the PCP site because (+)-**8a** did, in spite of their close structural similarity. More importantly, other (±)-2-substituted derivatives of (+)-**3** (Me, OH, OMe) have been shown by others to be significantly less active than their 3-substituted counterparts. The 3-substituted derivatives were at least equipotent with the parent compound, or showed even higher affinity as in the case of the 3-chloro and 3-bromo derivatives. This may indicate that steric bulk at the 2-position cannot be tolerated because it interferes with the binding to the receptor.

Recently, binding data on the *N*-(2-isothiocyanatoethyl) derivative of (±)-**3** were published by others. This compound showed wash-resistant inhibition of both the PCP site and the haloperidol-sensitive σ-receptor site in guinea pig brain, albeit at much higher concentrations (46% and 40% inhibition, respectively, at 100 μM) than (+)-**8a**. From structure-activity correlations and molecular modeling studies by other researchers it is clear that potent compounds based on (+)-**3** may only have small substituents on the nitrogen atom. This observation might explain why our *N*-(2-isothiocyanatoethyl) derivative of (±)-**3** is relatively inactive.

Computer-Assisted Molecular Modeling Studies (CAMM).

It is of interest to note that in (+)-**8a** [(+)-3-isothiocyanato-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*] cyclohepten-5,10-imine hydrochloride] the isothiocyanate group is positioned on aromatic ring B of the parent compound (+)-**3** (MK-801). This ring was not previously considered by others to be as important for binding as the A-ring in (+)-**3**. An aromatic ring in metaphit or etoxadrol-*meta*-isothiocyanate, which carries the isothiocyanate group, is essential for binding. If we assume that metaphit, etoxadrol-*meta*-isothiocyanate, and (+)-**8a** interact similarly with the PCP binding site, then it is likely that the aromatic rings of the three compounds are similarly located at the binding site and their isothiocyanate moiety interacts with either the same amino acid in the receptor at the binding site, or closely situated amino acids in three-dimensional space. Our comparison of structure-activity data for **3** and for PCP shows a resemblance which has not been taken into account in a previous study by others. Structure-activity studies on PCP show an almost complete loss of binding when the phenyl ring is substituted in the 4-position, while substitution with -OH or -NH_2 in the 3-position leads to increased affinity for the PCP site. A similar pattern of affinities is found for the 2- and 3-position of the B-ring in **3**. In contrast, substitution with OH or NH_2 in both the C7- and 8-positions of the other aromatic ring of **3** increases the in vitro binding by about two-fold. When the aromatic ring of PCP is overlapped with the B-ring of (+)-**3**, the 3- and 4-positions in PCP are in almost perfect overlap with the 3- and 2-positions in (+)-**3** (through CAMM, using Quanta/CHARMm), which is in agreement with the structure-activity data for these compounds. The alternate orientation shows limited overlap between the 7- and 8-positions of ring A

and the 4- and 3-positions, respectively, in PCP. Additionally, we have noted that the fit of (+)-**3** to the PCP pharmacophore is better when the B- instead of the A-ring is used as part of the pharmacophore. Other researchers have used a water molecule, positioned *syn* to the piperidine ring, as a model for the hydrogen bond acceptor group to which the protonated nitrogen atom binds. Our alternative orientation for PCP and derivatives in 3-dimensional space requires a more extended group such as a carboxylate as a hydrogen bond acceptor group to accommodate both PCP and (+)-**3**. Indeed, the PCP site has been shown by others to have acidic groups. We will address this in greater detail in future work.

Sigma Receptors

Synthesis and Biological Evaluation of 1-[1-(2-Benzo[b]thienyl)cyclohexyl]piperidine (BTCP) Homologues at Dopamine Uptake, Phencyclidine and Sigma Binding Sites. Piperidine and cyclohexyl ring homologues of the high affinity dopamine (DA) uptake inhibitor 1-[1-(2-benzo[b]thienyl)cyclohexyl] piperidine (BTCP, **3**) were each prepared in four steps from the appropriate cycloalkanones. These compounds were tested for their ability to displace [^{3}H]BTCP and [^{3}H]cocaine and to inhibit [^{3}H]DA uptake in rat striatal homogenates. The ratios IC_{50}[^{3}H]cocaine/IC_{50}[^{3}H]BTCP ranged from 62 for BTCP to 1.5 for 1-[2-(benzo[b]thienyl) cyclopentylamine (**17**); cocaine gave a ratio of 0.6. This indicates that BTCP is the most selective of all the compounds tested for sites labeled by [^{3}H]BTCP whereas cocaine is most selective for sites labeled by [^{3}H]cocaine. The wide differences in the relative abilities of these compounds to displace [^{3}H]BTCP and [^{3}H]cocaine suggests that these two radioligands are labeling different sites on the transporter. In general, the compounds structurally related to BTCP exhibited greater selectivity for sites labeled by [^{3}H]BTCP. However, several of the BTCP related derivatives showed greater (compared with BTCP and cocaine) ability to displace [^{3}H]cocaine. Most notably, 1-[1-(2-benzo[b]thienyl)cyclohexyl]pyrrolidine (**7**) exhibited a 3.4-fold greater affinity for these sites compared with BTCP and a 9-fold greater affinity at these sites than cocaine. Most of the BTCP homologues displayed greater ability to inhibit [^{3}H]DA uptake in rat forebrain synaptosomes than cocaine. BTCP and **7** were the most potent of all the compounds tested in terms of their ability to inhibit uptake of [^{3}H]DA. Ratios for IC_{50} [^{3}H]cocaine binding/IC_{50} [^{3}H]DA uptake ranged from 0.47 for 1-[1-(2-benzo[b]thienyl)cyclopentyl]homopiperidine (**11**) to 8.8 for 1-(2-benzo[b] thienyl)cyclohexylamine (**4**). The importance of this ratio is still unclear in terms of identification of potential cocaine antagonists. As for BTCP, all of the compounds tested showed K_i values > 10000 nM for displacement of [^{3}H]TCP from rat brain homogenates. These compounds were able to displace the highly selective sigma receptor probe, [^{3}H](+)-pentazocine from guinea pig brain homogenates with K_i values ranging from 125 nM to 9170 nM. The significance of their sigma binding activity in light of their dopaminergic properties is unclear. The diverse binding properties of these compounds at the DA-uptake site and their spectrum of inhibitory activities for [^{3}H]DA-uptake identifies them as a useful base for the development of subtype selective probes at this site. These compounds will allow further study of the structure and function of the "cocaine" receptor as well as the development of potential cocaine antagonists.

Synthesis, Configuration and Binding of Isomeric 2-Phenyl-2-(N-piperidinyl)bicyclo[3.1.0] hexanes at Phencyclidine and Sigma (σ) Binding Sites. The novel semirigid derivatives (+)-*cis*-1-[2-phenyl-2-bicyclo[3.1.0] hexyl]piperidine [(+)-**8**], its enantiomer (-)-**8** and (±)-*trans*-1-[2-phenyl-2-bicyclo[3.1.0]hexyl]piperidine [(±)-**9**] were synthesized as probes to investigate the mode of interaction of phencyclidine (PCP) with its binding site on the N-methyl-D-aspartate receptor complex. Each target compound was obtained in 5 steps starting from cyclopent-2-enone. (+)- and (-)-**8** were obtained in greater than 98% optical purity through 3 recrystallizations from ethanol of the *S*-(+)- and *R*-(-)-mandelate salts of intermediate (±)-cis-2-phenyl-2-bicyclo[3.1.0]hexylamine [(±)-**16**]. Crystallization of the *R*-(-)-mandelate salt afforded 1*R*,2*R*,5*S*-(-)-**16** whereas the *S*-(+)-mandelate salt afforded 1*S*,2*S*,5*R*-(+)-**16**; the absolute configuration was determined by single crystal X-ray analysis of (-)-**16**•R-(-)-mandelate. Single crystal X-ray analysis of (±)-**9**•picrate confirmed its *trans* configuration and provided conformational data.

(+)- and (-)-**8**, (±)-**9** were examined for their ability to interact with PCP and σ binding sites in vitro using [^{3}H]TCP and [^{3}H]pentazocine as radioligands. The binding was compared with that of PCP and contrasted with the rigid symmetrical phencyclidine derivatives *cis*- and *trans*-1-[3-phenyl-3-bicyclo[3.1.0]hexyl]piperidines (**6** and **7**). The results of the study indicated that the conformations of PCP represented by **6-9** are not optimal for potent interaction at either of these sites. Affinities ranged from 582 nM [(±)-**8**] to 29000 nM [(+)-**8**] at PCP binding sites and from 1130 nM [(-)-**8**] to 16300 nM (**7**) at σ sites. In this assay, PCP exhibited affinities of 64.5 nM at PCP and 1090 nM at σ sites. Qualitative correlation between the σ and PCP binding data suggests some similarities between these binding sites.

An axial phenyl and equatorial piperidine ring with the nitrogen lone pair of electrons antiperiplanar to the phenyl ring has been postulated as the receptor active conformation of PCP-like ligands at the PCP binding site. Comparison of the binding data of **7-9** with that of the previously described methylcyclohexylPCP derivatives allowed its rationalization in terms of this model. It is likely that the lowered affinity in this bicyclo[3.1.0]hexane series is a consequence of non-optimal geometry (pseudoequatorial phenyl or pseudoboat) for binding as opposed to the presence of steric bulk which proved deleterious in the methylcyclohexylPCP derivatives.

Synthesis of N^1-3-[^{18}F]Fluoropropyl-N^4-2-([3,4-dichlorophenyl]ethyl)piperazine, a High Affinity Ligand for Sigma Receptors. The radiochemical synthesis of the high affinity, selective sigma receptor ligand, N^1-3-[^{18}F]fluoropropyl-N^4-2-([3,4-dichlorophenyl]ethyl)piperazine, was reported. The labeled compound was prepared by fluoride displacement on a bis-methanesulfonate salt of the propylmethanesulfonyloxy precursor. The product was isolated by first passage through a BONDELUT-SI and subsequently HPLC in high radiochemical and chemical purity with a decay corrected radiochemical yield of 49±9%.

Immunoregulatory Properties of (+)-Pentazocine and Sigma Ligands. Pentazocine, phencyclidine, and other sigma ligands including 1,3-di-(*o*)tolylguanidine (DTG),(+)-1-propyl-3-(3-hydroxyphenylpiperidine [(+)-PPP] and haloperidol were investigated for their potential immunoregulatory properties. High concentrations (10^{-5} M) of DTG and haloperidol were found to suppress in vitro murine splenocyte natural killer activity while equivalent concentrations of (+)-pentazocine, (-)-pentazocine and (+)-PPP were without effect. In a reciprocal fashion lower doses (10^{-9} M) of DTG enhanced natural killer activity. Sigma ligands were also found to affect in vitro polyclonal immunoglobulin production following mitogen stimulation. Specifically, high concentrations (10^{-6} M) of haloperidol significantly ($P<0.001$) suppressed pokeweed mitogen PWM-stimulated IgG and IgM production, yet enhanced lipopolysaccharide (LPS)-stimulated IgM production by murine splenocytes. Lower concentrations (10^{-8} to 10^{-10} M) enhanced (two- to fourfold) PWM-induced IgM production and LPS-stimulated IgG and IgM production at high concentrations (10^{-6}), (+)-pentazocine suppressed ($P<0.01$) LPS-induced polyclonal IgG and IgM but enhanced ($P<0.01$) PWM-induced IgM production. Both DTG and (+)-pentazocine (10^{-8} to 10^{-10} M) significantly augmented (two- to threefold) LPS-stimulated murine splenocyte production of polyclonal IgM. Intracellularly, (-)-pentazocine (10^{-9} M), haloperidol (10^{-6} M) DTG (10^{-7} M) and (+)-PPP (10^{-5} to 10^{-9} M) enhanced forskalin (10^{-6} M)-induced cAMP production in splenic lymphocytes while (+)-pentazocine was without effect. Collectively, the data suggest functional and biologically relevant sigma receptors on cells of the immune system.

Receptor Biochemistry and Pharmacology Unit of the Drug Design and Synthesis Section

Characterization Of Novel Synthetic Sigma Ligands (W. Williams, B.J. Vilner). We continue to develop ligands with higher sigma receptor affinity and selectivity. Several novel ligands were synthesized by chemists in the Drug Design and Synthesis Section, LMC (B.R. de Costa, X.-S. He, C. Dominguez, L. Radesca), and screened for sigma subtype affinity in the Unit on Receptor Biochemistry and Pharmacology. Ligands of the aryl ethylene diamine, aryl piperazine, and alkyl aryl polyamine classes were characterized as having extremely high sigma affinity, in some cases in the subnanomolar range. All of these ligands contain a critical sigma pharmacophore which may consist of

N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)ethylamine as a nucleus. These compounds have been found to act as sigma agonists or antagonists in functional assays (see below). Benzomorphan-related ligands were synthesized at Research Triangle Institute (F.I. Carroll). Several high affinity compounds were identified with low nanomolar affinity, for example (+)-N-benzyl-normetazocine (0.67 nM at sigma-1 sites). Enantiomeric benzomorphans were used to verify the subtype classification of sigma sites, since sigma-1 sites exhibit higher affinity for the (+)-enantiomer whereas sigma-2 sites show preference for the (-)-enantiomer.

Heterologous Modulation Of Phosphoinositide Turnover By Sigma Receptors (J.M. Cutts, K.K. Hsu). Little is known of the signal transduction mechanism(s) utilized by sigma receptors. We have shown that sigma ligands negatively modulate the ability of muscarinic cholinergic agonists and α-1 adrenergic agonists to stimulate phosphoinositide turnover in rat brain. This is mediated by sigma-1 receptors and results in a reduction in the maximal stimulation produced by muscarinic or adrenergic agonists. Sigma-1 involvement was confirmed by the finding that sigma ligands show less efficacy in cell lines with a low density of sigma-1 sites. Studies are currently underway to investigate the mechanism by which sigma ligands produce this effect. Evidence to date suggests a sigma receptor mediated reduction in the number of muscarinic receptors. This could have important functional implications, as sigma ligands would be expected to have anticholinergic properties in behavioral or physiological paradigms.

Development Of Novel Sigma Agonists And Antagonists (K.K. Hsu, J.M. Cutts). Sigma inhibition of the muscarinic phosphoinositide (PPI) response was used as a functional assay to screen novel synthetic sigma compounds. Despite high sigma binding affinity, several novel aryl ethylene diamines, aryl piperazines, and alkyl aryl polyamines exhibited lower than expected efficacy, suggesting that they might be antagonists or partial agonists at sigma-1 sites. BD1139 was shown to inhibit the PPI response at concentrations above 50 uM, but had no effect at lower concentrations. At 10, 30, and 50 uM, BD1139 dose-dependently attenuated the ability of (+)-pentazocine and SH311 to inhibit oxotremorine-M-stimulated PPI turnover. These results are consistent with this compound being a partial agonist. Two other compounds, BD1047 and BD1063 were identified and are being investigated as possible full sigma-1 antagonists. These compounds will be extremely useful for functional studies of sigma sites and may have potential therapeutic value for treating motor disorders (see below).

Morphological And Cytotoxic Effects Of Sigma Compounds (B.J. Vilner). In the course of investigating the behavioral effects of haloperidol and its metabolites by microinjection into the red nucleus of the rat, we discovered that reduced haloperidol (a major metabolite in humans and a potent sigma ligand) was neurotoxic. The compound caused extensive gliosis and loss of magnocellular neurons in and around the area of microinjection. These pathologic changes were accompanied by long-lasting (> 3 days) postural abnormalities. Similar results were observed with BD614, a novel and highly selective sigma ligand. It was difficult, initially, to link these effects with action at sigma receptors since other sigma ligands tested had produced only short-lived (90 min) postural effects with no obvious signs of histological damage.

In order to examine the possible involvement of sigma binding sites in this cytotoxic effect, we initiated a study of the effects of other neuroleptics and various other sigma ligands on clonal cell lines. We have shown that sigma-1 and sigma-2 sites are present in several clonal cell lines, including NB41A3, N1E-115, and S20-Y neuroblastomas, C6 glioma, and the NG108-15 neuroblastoma-glioma hybrid cell line. This suggested that these cells could serve as appropriate model systems in which to study sigma receptor function. C6 glioma cells were used for further study.

Typical neuroleptics which exhibited sigma binding affinity caused withdrawal of processes, rounding, and ultimately cell death upon continued exposure. Some compounds (100 μM) produced noticeable effects in less than six hours of exposure, and all produced effects by 24 hours. Among these are haloperidol, reduced haloperidol, trifluperidol, fluphenazine, perphenazine, and thioridazine. Potency correlated generally with rank order of affinity at sigma sites. The atypical neuroleptic (-)-sulperide which lacks sigma binding affinity had no effect on cells. Also, agonists and antagonists for dopamine, serotonin, adrenergic, glutamate, GABA, PCP, opiate, and muscarinic cholinergic receptors which lack affinity for sigma sites failed to have significant effect on cells in up to 72 hours of culture at concentrations up to 300 μM. The results support the notion that the effects

are mediated by sigma receptors. Specifically, the effects appear to be mediated by a novel subtype of sigma-1 site identified in C6 glioma and other cell lines.

The results suggest that sigma receptors, which are present in high concentrations in brain motor nuclei, might mediate toxic alterations in cells upon chronic exposure to neuroleptics and/or their sigma-active metabolites. This might in turn contribute to the latent and irreversible neuroleptic-induced motor abnormalities such as tardive dyskinesia. The data also suggest a possible role of sigma receptors in idiopathic neurodegenerative disorders. Thus, sigma receptor antagonists might be useful in treating or preventing both drug-induced and idiopathic motor disturbances. Studies of other sigma compounds and the mechanism of this effect are underway.

Ligands For In Vivo Receptor Imaging (B.J. Vilner; W. Williams). Fluorinated and iodinated aryl ethylene diamines were synthesized in LMC (X.-S. He) as potential PET and SPECT ligands for visualizing sigma sites in functioning animals. These compounds maintained high affinity at sigma-1 and sigma-2 receptors. SH344 (Ki = 2.5 nM and 43.1 nM at sigma-1 and sigma-2, respectively) was synthesized in radiolabeled form using ^{123}I and ^{125}I (K.-S. Lee, NIMH). The ^{123}I-labeled compound exhibited 97% specific binding to guinea pig brain membranes. Preliminary SPECT imaging studies in rhesus monkeys shows that the compound readily enters the brain, has a long wash-out time, and visualizes brain regions. Studies are currently underway to characterize the in vitro binding properties of ^{125}I-labeled SH344. This compound shows promise as both a diagnostic tool and as a probe for further study of sigma receptor biochemistry.

Isolation, Purification, And Molecular Characterization Of Sigma Receptors (C. Torrence-Campbell): A project was initiated with the goal of purifying sigma receptors for the purpose of molecular characterization and ultimate cloning. Rat liver is a rich source of both the sigma-1 and sigma-2 subtypes and was therefore chosen as the initial receptor source. Receptors have been solubilized in active form with CHAPS. Both subtypes are present in the extracts, however there is preliminary evidence of preferential solubilization of sigma-1 sites. Several novel amine ligands with high affinity and selectivity for sigma receptors are available for column derivatization, and we plan to use affinity chromatography as a major tool to differentially isolate sigma-1 and sigma-2 sites. Furthermore, the highly sensitive probe [^{125}I]SH344 (discussed above) should be useful in attempts to clone the receptor by expression methodology.

Behavioral Pharmacology Unit of the Drug Design and Synthesis Section

Behavioral Effects of Cocaine and Other Drugs of Abuse. The primary current effort of the lab is to characterize the effects of GBR & sigma ligands using the cocaine self-administration in monkeys. In these studies lever-press responding of 6-10 kg male rhesus monkeys is typically maintained under multiple FR 30-response schedules of food and intravenous cocaine delivery. Relatively low doses (3-10 µg/kg/inj) of cocaine maintain high rates of responding in the drug delivery components, and do not affect responding maintained by food presentation. Under these conditions, the effects of GBR 12909HCl and GBR 12935HCl (0.3-5.6 mg/kg, i.v. slow infusion) were compared. Both agents selectively decreased cocaine-maintained responding within a limited range of doses, while lower doses had no effect and higher doses decreased responding in both components. These results suggest that these GBR analogs can selectively attenuate cocaine self-administration, without affecting alternative behaviors. However, these agents may not exhibit full agonist (cocaine-like) effects. In several additional studies, we have been unable to maintain drug-seeking behavior with GBR in cocaine-naive monkeys, in contrast to previous reports of maintenance under substitution paradigms where monkeys have a history of self-administration. These results suggest that GBR-based agents may be useful in the development of drugs to treat cocaine abuse. Additional studies have been directed at the link between susceptibility to drug abuse and activation of the hypothalamic pituitary adrenal (HPA) axis. Two lines of studies have pursued this association, one in monkeys and the other in rodents.

Behavioral Effects of Stress-Related Peptides and Hormones - Primate Studies. The primary endogenous agents involves in the HPA axis are corticotropin releasing hormone (CRH), adrenocorticotrophic hormone and cortisol. Several studies have been directed at examining the behavioral specificity of the direct effects of CRH on behavior. In order to accomplish this, a unique

i.c.v. delivery device had to be designed, constructed and tested. This device was designed to allow the delivery of drugs or other agents to discrete loci within the CNS of animals while maintaining sterile conditions. It was an improvement over existing designs because it: 1) maintains an absolute minimal dead space within the system, 2) is smaller in diameter (by approximately 80%) than existing shunt catheters, minimizing tissue damage during placement, 3) is easily secured and requires minimal clearance over the cranium, and 4) maintains a sterile seal between the brain and periphery. Preliminary studies indicate the device is well accepted and is fully functional for periods up to a year. The device is intended for permanent implantation. Its efficiency has been demonstrated through several published reports of the effects of CRH or related agents on primate behavior.

For example, in one, the behavioral consequences of the central administration of CRH in rhesus monkeys was determined using food-maintained behavior. Acute doses of CRH (0.003 ng/kg-10 µg/kg, i.c.v.), decreased responding for food in a dose- and time-related manner. With intermediate doses, responding occurred at a high rate until food was delivered, and then ceased abruptly for several minutes. Previous studies have attributed similar effects to the noxious properties of certain drugs. Acute doses had no effect on home cage food consumption, body weight, or responding for food on subsequent days. When CRH was given repeatedly for several days, its behavioral suppressant effects increased. Home cage food intake, body weight, and subsequent responding for food decreased for up to six weeks before returning to normal. These results suggest that sustained elevations in central levels of CRH can result in a sensitization to its anorexigenic effects, an effect that has not been reported in other species. Because hyperaroused clinical states such as depression, anorexia nervosa, and some forms of drug abuse are characterized biochemically by hypercortisolism and elevated CRH in CSF, these anorexigenic effects may corroborate a potential role for CRH in affective disorders were comorbidity with drug abuse is high.

In another study, rhesus monkeys were equipped with the i.c.v. cannula system and trained to respond under operant schedules of food presentation or from stimuli associated with escape. CRH decreased food-maintained behavior in a dose-related manner over the range of (0.3-10 µg/kg) but did not affect escape responding, demonstrating a selective effect on food-maintained responding. This selective effect was related to the tendency for responding to stop after delivery of a food pellet when higher does of CRH were given, consistent with the notion that a conditioned aversion to food was established in the presence of CRH. This may suggest that in hyperaroused clinical states such as depression and anorexia nervosa, focus is shifted away from appetitive tasks as a result of increased levels of CRH.

Several additional studies that were directed at determining the nature of the behavioral specificity of the effects of the CRH peptide were also conducted, and incorporated into a recently published review. This review focuses on those aspects of the behavioral effects of CRH related to food-associated behaviors. The effects of CRH on food intake are compared with its effects on performances maintained by food presentation, and contrasted with the effects of CRH on performances maintained by other events. The effects of CRH antagonists and drugs which interact with the behavioral effects of CRH are also reviewed, particularly with respect to their direct effects on food intake. Lastly, data assessing the effects of CRH administration on central neurotransmitter levels are presented and compared with levels seen in clinical populations. The effect of CRH on food intake seen in animals is consistent with a putative role for CRH in clinical syndromes where appetite suppression is apparent. Since some of the effects of CRH on food intake are subject to pharmacological intervention, strategies directed at peptidergic mechanisms of drug-abuse associated disorders should be explored.

Several additional behavioral studies have been directed at the association between particular behavioral effects a drug of abuse, such as cocaine, and the subsequent tendencies to abuse or avoid further drug contact, in order to further elucidate the role of stress in drug abuse. In one set of experiments, monkeys were trained to respond under second-order schedules of food presentation and then exposed to either a self-administration (SA) or to a conditioned taste aversion (CTA) procedure. Initial exposure to stimuli associated with post-session administration of 0.3 mg/kg cocaine either maintained (SA) or suppressed (CTA) responding, respectively. The monkeys were then exposed to the alternate procedure. Initial exposure to CTA, decreased cocaine SA responding compared to rates seen with initial SA exposure. In contrast, with initial exposure to SA, the CTA procedure failed to suppress responding. Thus, prior exposure to either reinforcing or suppressant effects of cocaine altered the subsequent behavioral effects of that drug, suggesting a unique role of behavioral history in the abuse potential of cocaine.

Individual Differences in Pharmacological and/or Behavioral Effects of Treatment in Rodents - Neuroendocrine and Behavioral Interaction. A second line of studies furthers the association between stress and drug abuse using rodent models. For example, different rat strains can exhibit large differences in hypothalamic pituitary-adrenal activity that have been used to determine the role of the neuroendocrine system in susceptibility to autoimmune disease, drug abuse and other behavioral end points. To further characterize potential behavioral correlates of these differences, the amplitude of the acoustic (ASR) and tactile (TSR) startle response and the corticosterone response to acoustic startle stimuli were compared between two histocompatible strains, Lewis (LEW/N) and Fischer (F344/N) rats, as well as outbred Harlan Sprague-Dawley (SD) rats. The startle response to intense environmental stimuli is found in most, if not all, animal species. Startle stimuli elicited larger ASR and TSR in LEW/N rats than in F344/N rats, with SD rats exhibiting an intermediate response. The ASR habituated at a similar rate in LEW/N and F344 rats, while the ASR did not habituate in SD rats. After handling and placement in the startle chambers, the three strains did not differ in control levels of corticosterone. In contrast, exposure to acoustic startle stimuli increased corticosterone 5-fold in F344/N rats and 2-fold in SD rats, but had no effect on corticosterone in LEW/N rats. These findings suggested an inverse relationship between the amplitude of the ASR and hypothalamic-pituitary-adrenal activation across strains. This relationship was further supported by a high negative correlation between corticosterone level and ASR amplitude within the F344/N group. These studies have been extended by comparing the amplitude of the startle response among forty-six inbred and outbred rat strains. These strains exhibited differences in both the mean amplitude of the startle response and the rate of habituation to startle stimuli over repeated trials. In addition, there was a significant relationship between these measures. These results suggest that robust phenotypic differences in startle response exist among rat strains. As previous investigations have demonstrated a direct relationship between the startle response and other behavioral end points, the use of strain differences in startle response may be an effective way to determine genetic contributions to specific behavioral responses.

It has also been well-established that challenge with inflammatory stimuli, stressors, or specific drugs render LEW/N rats susceptible to autoimmune disease while their histocompatible control, the F344 rat, is resistant. In order to examine behavioral correlates of suspected differences in hypothalamic pituitary adrenal mechanisms responsible for that effect in these strains, both strains were a) assessed for differences in behavioral and corticosterone responses to exposure to an open field, b) prepared with ventricular cannuli, and assessed again in the open field after saline, or c) after 3 μg/rat of CRH. Significant baseline differences in open field response (pattern of ambulation), and in the effects of CRH on rearing, grooming, and activity were found between these strains. These differences suggest that differences in endogenous CRH may form the basis for the differential susceptibility of these strains to autoimmune disease. Such differences may serve as an animal model for genetic determinants of relationships between CNS function and the immune system.

Lastly, since recent studies have found the LEW/N rat self-administers drugs of abuse at higher rates than the F344/N rat, suggesting a genetic predisposition toward the abuse potential of drugs, another study compared the acquisition of a conditioned taste aversion (CTA) to cocaine in these strains. During an initial 20-min daily session a 0.1% saccharin solution was available and a dose (0-50 mg/kg, sc.) of cocaine was given immediately after that session. Water was available during sessions on the following three days. Fluid consumption was assessed over three saccharin/water cycles, and a final saccharin session. Vehicle injections (0 mg/kg) that followed exposure to saccharin had no effect on subsequent saccharin consumption. In contrast, when cocaine followed exposure to saccharin, rates of saccharin consumption decreased over successive saccharin sessions in a dose-related manner in both strains. The lowest dose (18 mg/kg) decreased consumption in LEW/N rats but not in F344/N rats. An intermediate dose (32 mg/kg) decreased consumption maximally in LEW/N rats, and only marginally in F344/N rats. The highest dose (50 mg/kg) decreased consumption completely in LEW/N rats, and almost completely in F344/N rats. These findings demonstrate that significant differences in sensitivity to stimuli paired with cocaine occur between these strains. These differences are consistent with previous reports that the LEW/N rat is uniquely sensitive to both behavioral and biochemical effects of drugs of abuse. The current report extends this sensitivity to the noxious effects of these drugs. To the extent that noxious and reinforcing effects of cocaine are unrelated, these results suggest that the LEW/N rat does not exhibit a genetic predisposition to factors related only to the abuse potential of drugs.

Adverse Consequences of Exposure to Drugs of Abuse: Behavioral Toxicology and Risk Assessment. Drugs of abuse exhibit adverse effects which are poorly understood. In order to more fully characterize the range of doses over which these effects can be observed, and predict neurotoxic liability, several different agents were studies for the ability to impair normal behavior. In one experiment, four homologous *n*-alkanes were compared for their ability to impair performance and stimulate hypothalamic-pituitary activity in mice. Performance was assessed using operant responding maintained under an FI 60-sec schedule of milk presentation. Cumulative concentration-effect functions for octane, heptane, hexane and pentane were obtained by incrementally increasing exposure concentrations until responding was abolished. Recovery from these rate-decreasing effects was determined 30 min after exposure to the highest concentration. Rate-decreasing potency (EC50) was greatest for octane (2474 ppm), and progressively less for heptane (3872 ppm), hexane (7051 ppm), and pentane (36130 ppm). Responding recovered completely, 30 min after exposure, for pentane and hexane, to 75% of pre-exposure levels for heptane, but to only 15% of pre-exposure levels for octane. The risk of obtaining a small effect with these agents (the concentration expected to decrease performance 10% in one out of one thousand mice) exhibited a similar order. The effect was predicted to occur at 227 ppm for octane, 331 ppm for heptane, and 1429 ppm for pentane. However, this prediction occurred at an unusually low dose for hexane (68 ppm). These *n*-alkanes also stimulated up to 2000-fold increases in adrenocorticotropin hormone (ACTH) release. *n*-Hexane was slightly more potent and produced larger effects. These studies demonstrate a direct relationship between aliphatic carbon chain length and the potency of *n*-alkanes in impairing performance.

In another study, the behavioral and neuroendocrine correlates of diethyl ether exposure were compared in the mouse. Diethyl ether has anesthetic, neuroendocrine-stimulating, and abuse-potential properties, yet little is known of the concentrations over which these apparently diverse behavioral effects occur. Adult male NIH mice were exposed to a range of concentrations of ether (1000-30000 ppm) in order to characterize it effects on operant behavior and neuroendocrine activity. When responding was maintained under FI-60 sec schedules of milk presentation, 5- or 30-min exposures to less than 3000 ppm ether were without behavioral effect, 10000 ppm produced up to 300% increases in responding, and higher concentrations abolished responding. Exposure to a similar range of concentrations elevated adrenocorticotrophic hormone (ACTH) and corticosterone levels in a dose- and time-dependent manner. Short (5 min) exposures elevated baseline levels of ACTH (18.2 pg/ml) to 310.5 pg/ml (~1700% of control) at 10000 ppm, whereas corticosterone was relatively unaffected. With 30 min of exposure to 10000 ppm ether, corticosterone increased maximally from 78.44 ng/ml to 559 ng/ml (~700% of control) and ACTH was increased to a lesser extent. The imidazobenzo-diazepine, Ro 15-4513, decreased FI responding at doses greater than 3 mg/kg and attenuated the rate-increasing effects of diethyl ether at 1 mg/kg. Several additional reports and chapters have issued from this general grouping of work, indicating the need to develop risk assessment protocols more fully.

Behavioral Effects of Sigma Ligands. Excitatory amino acids, acting at the NMDA and/or sigma receptors, have been postulated to play an important role in the acquisition of some behavioral effects of drugs, in particular the development of tolerance. Drugs that block this receptor also attenuate many of the effects associated with learning and memory, suggesting a significant behavioral component to addictive processes. We previously reported that excitatory amino acid antagonist, MK-801, blocked the acquisition of a conditioned emotional response (CER), consistent with the notion that excitatory amino acid blockade prevented the development of a learned emotional response. Current studies focus on the role of long-term treatment with antipsychotic medication, as debilitating motor side effects (tardive dyskinesia) occur in humans. These effects are thought to arise from long-term blockade of dopamine (DA) receptors. In rats, this side effect is quantified as catalepsy, and is reliably induced by haloperidol. In vitro and in vivo studies have suggested that the sigma ligands are able to suppress / reverse the motor side effects which would otherwise occur as a consequence of neuroleptic (antipsychotic) administration. To test this hypothesis, we have constructed catalepsy dose-response curves with dopaminergic ligands such as haloperidol, and are testing the ability of known sigma ligands (e.g.(+)-pentazocine, DTG, dextromethorphan) to shift the dose-response curve to the right. If these tests are viable, we will extend the test to other untested sigma ligands, including the BD-series ligands, and GBR-related compounds. If the relative affinities and catalepsy-shifting effects remain concordant, other neuroleptics such as chlorpromazine and remoxipride will be tested.

BIOMEDICAL CHEMISTRY SECTION

Targeting RNA For Degradation With A 2',5' Oligoadenylate-Antisense Chimera. Antisense oligonucleotides hold considerable promise both as research tools for inhibiting gene expression and as agents for the treatment of a myriad of human diseases. However, targeted destruction of RNA has been difficult to achieve in a versatile, efficient and reliable manner. We have developed a novel and effective strategy for cleaving unique RNA sequences with 2-5A-dependent RNase, an endoribonuclease which mediates inhibitory effects of interferon on virus infection, and is activated by 5'-phosphorylated, 2',5'-linked oligoadenylates known as 2-5A, [p_n5'A2'(p5'A2')$_m$p5'A], resulting in the cleavage of single-stranded RNA predominantly after UpUp and UpAp sequences. To direct 2-5A-dependent RNase to cleave unique RNA sequences, p5'A2'p5'A2'p5'A2'p5'A was covalently linked to antisense oligonucleotide to yield a chimeric molecule (2-5A:AS). The antisense oligonucleotide component of 2-5A:AS bound a specific RNA sequence while the accompanying 2-5A component activated 2-5A-dependent RNase thereby causing the cleavage of the RNA in the targeted sequence. This strategy was demonstrated by inducing specific cleavage within a modified HIV-1 vif mRNA in a cell-free system from human lymphoblastoid cells. Because 2-5A-dependent RNase is present in most mammalian cells, the control of gene expression, including therapies for cancer, viral infections, and certain genetic diseases, can be envisioned based on this technology.

Synthesis And Phamacokinetics Of A Dihydropyridine Chemical Delivery System For The Antiimmunodeficiency Virus Agent Dideoxycytidine. In order to explore the possibility that a dihydropyridine/pyridinium redox chemical delivery system might enhance significantly the brain uptake of the anti-HIV agent dideoxycytidine (DDC), we prepared a DDC derivative which bore the 1,4-dihydro-1-methyl-3-pyridylcarbonyl moiety at both the cytidine exocyclic amino moiety and the sugar 5'-hydroxyl function; namely, 5',4N-bis(1,4-dihydro-1-methyl-3-pyridinylcarbonyl)-2',3'-dideoxy-cytidine (**2**). In cell-free extracts of rat brain tissue, compound **2** was readily converted to free DDC by stepwise oxidation and hydrolysis of the dihydropyridyl groups. Time-dependent plasma and brain concentrations of DDC and **2** were determined following i.v. administration of **2** (49.3 mg/kg) to rats. Compound **2** could be detected in brain, reaching peak concentrations of 7.7 ± 2.9 nmol/g at 15 min. Low levels of DDC also were detected with a peak concentration of 1.4 ± 0.5 nmol/g at 240 min after injection. The brain/plasma concentration integral of compound **2** was 0.95 whereas that for DDC in brain as a ratio of combined DDC and compound **2** levels in plasma was 0.24. Despite this, brain concentrations remained low and not significantly different from those achieved following administration of DDC alone.

Fluorodeoxy Sugar Analogues Of 2',5'-Oligoadenylates As Probes Of Hydrogen Bonding In Enzymes Of The 2-5A-System. In order to understand the contribution of the 3'-hydroxyl groups of 2-5A$_4$ (ppp5'(A2'p)nA) to its interaction with RNase L, we synthesized a series of analogues in which the 3'-hydroxyl moiety was replaced by fluorine to yield 3'-fluoro-3'-deoxyadenosine (A_F). These included: ppp5'A2'p5'A2'p5'A_F, ppp5'A2'p5'A_F2'p5'A, ppp5'A_F2'p5'A2'p5'A, ppp5'A_F2'p5'A_F2'p5'A_F, ppp5'A2'p5'A_F2'p5'A_F, ppp5'A_F2'p5'A2'p5'A_F, and the corresponding monophosphates. When these oligomers were evaluated for their ability to activate RNase L from various sources, we found that the replacement of the second from the 5'-terminus adenosine residue of 2-5A with the fluoro analogue caused major reductions in activity. We conclude that the hydroxyl group of this second or middle nucleotide residue of 2-5A trimer acts as a hydrogen bond donor to an acceptor group in RNase L and that this hydrogen bond is key to what we presume may be a critical conformational change required for nuclease activity of RNase L. We have also found that substitution of fluorine for hydroxyl in the second or penultimate residue of 2-5A trimer results in an oligomer with a 2',5'-phosphodiesterase sensitivity comparable to 2-5A itself. When viewed in terms of earlier experiments, these results imply that the role of the 3'OH group of the penultimate nucleotide of 2-5A may be to anchor the substrate to the phosphodiesterase through its action as a hydrogen bond receptor.

SECTION ON CARBOHYDRATES

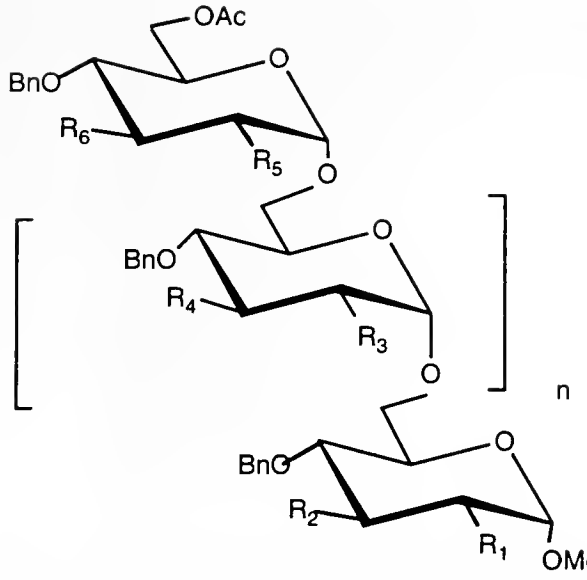
Syntheses of Methyl α-Isomalto-Oligosaccharides Specifically Deoxygenated at Position 2. This Section has studied the binding of carbohydrate ligands to monoclonal antigalactan and antidextran antibodies using mono- and oligosaccharides, and their deoxy and deoxyfluoro analogues, respectively. Changes in binding due to replacement of hydroxyl group by fluorine or hydrogen in saccharide can distinguish if H-bonding comes from the protein to the ligand or vice versa. We are studying anti-dextran IgG 35.8.2H, isolated by Kabat and co-workers. It is an anti (1,6)-dextran capable of binding internal antigenic epitopes. In our binding studies we used methyl α-D-glucopyranoside and methyl α-glycosides of isomalto-oligosaccharides from the di- to the octasaccharide as ligands. Using ligand-induced protein fluorescence change, the K_a values were determined. A fluorescence change was observed with the methyl α-D-isomaltotrioside and higher oligosaccharides, but not with methyl α-D-glucopyranoside or methyl α-isomaltoside. This suggests the preparation of methyl α-D-triosides specifically deoxygenated at different positions which we report here.

We present a procedure for the preparation of methyl α-isomalto-oligosaccharides (Figure 1) specifically deoxygenated at C-2. We have used nucleophiles derived from methyl α-D-glucopyranoside. They can be deoxygenated at position 2 and appropriately substituted at the other carbons, while keeping C-6 unsubstituted, the position to be condensed with the donor. Methyl 2,3,4-tri-O-benzyl-α-D-glucopyranoside (**1**), one of the possible nucleophiles, was prepared as we previously described. The other nucleophile, methyl 3-O-benzoyl-4-O-benzyl-2-deoxy-α-D-arabinohexopyranoside (**2**), as well as the corresponding glycosyl donor 6-O-acetyl-3-O-benzoyl-4-O-benzyl-1-O-tertiarybutyl(dimethyl)silyl-2-deoxy-β-D-arabinohexopyranose (**4**), was prepared as in Scheme 1: Methyl 4,6-O-benzylidene-α-D-glucopyranoside was converted (73%) into the selectively phenoxythiocarbonylated derivative (**5**). After benzoylation (94%) the reduction with tributyltin hydride in the presence of 2,2'-azobis(2-methylpropionitrile) smoothly afforded methyl 3-O-benzoyl-4,6-O-benzylidene-2-deoxy-α-D-arabinohexopyranoside (**7**) (91%). The opening of 4,6-O-benzylidene ring with borane trimethylamine complex and aluminium chloride in toluene then afforded the nucleophile **2** (85%). This was treated with sulfuric acid in acetic anhydride to give 1,6-di-O-acetyl-3-O-benzoyl-4-O-benzyl-α-D-arabinohexopyranose (**9**) in 95% yield. Subsequent selective deacetylation of di-O-acetyl derivative with tributyltin ethoxide in toluene gave 6-O-acetyl-3-O-benzoyl-4-O-benzyl-2-deoxy-α,β-D-arabinohexopyranose (**10**) (85%) which, after silylation with *t*-butyl(dimethyl)silyl chloride in the presence of imidazole yielded the glycosyl donor **4** (85%). Finally the second donor used, 6-O-acetyl-2,3,4-tri-O-benzyl-α-D-glucopyranosyl chloride (**3**) was prepared as described. The condensation of the tertiary-butyl(dimethyl)silyl derivative **4** with methyl 2,3,4-tri-O-benzyl-α-D-glucopyranoside (**1**) (trimethylsilyltriflate) afforded methyl O-(6-O-acetyl-3-O-benzoyl-4-O-benzyl-2-deoxy-α-D-arabinohexopyranosyl)-(1-6)-2,3,4-tri-O-benzyl-α-D-glucopyranoside (**11**) (Scheme 2a). The 6-O-acetyl group in **11** could be easily removed in selective fashion. Thus, the reaction of **11** with hydrogen chloride in methanol gave nucleophile **12** (90%) which was used for various glycosidation reactions.

The condensation of hydroxyl derivative **12** with silyl derivative **4** (trimethylsilyl triflate) gave trisaccharide **13** (80 %). The trisaccharide **14** (85%) was prepared using the same nucleophile (**12**) and 6-O-acetyl-2,3,4-tri-O-benzyl-α-D-glucopyranosyl chloride (**3**) (silver carbonate, silver triflate)(Scheme 2b).

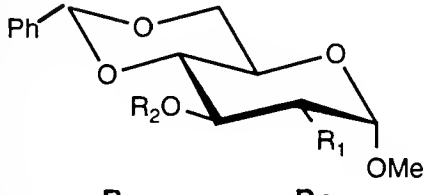
The condensation of methyl glycoside **2** with *t*-butyl(dimethyl)silyl derivative **4** (trimethylsilyl triflate) to afford the α-glycoside **15** in 88% yield. Disaccharide **16** was prepared using chloride **3** and nucleophile **2** (silver carbonate, silver triflate) (Scheme 2a).

Figure 1 (next page)

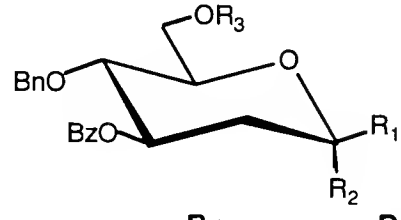


Where R_1, R_3, R_5 can be either Bn or H and R_2, R_4 and R_6 can be either Bn or Bz.

Scheme 1

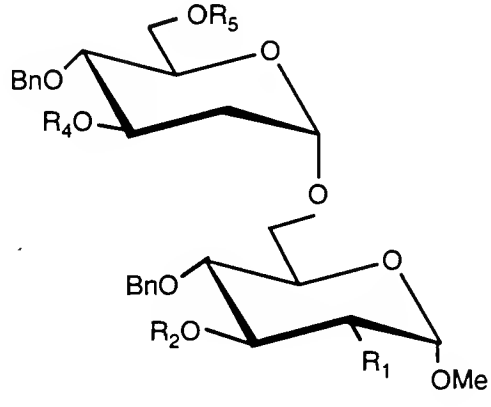


	R_1	R_2
5	OPtc	H
6	OPtc	Bz
7	H	Bz



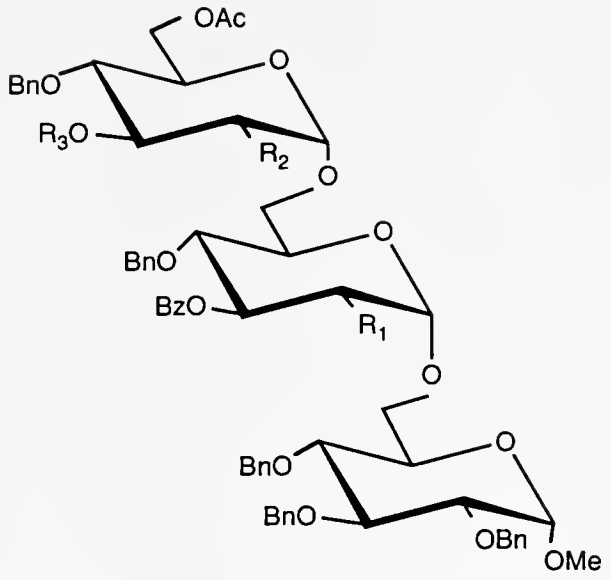
	R_1	R_2	R_3
2	H	OMe	H
9	H	OAc	Ac
10	H,OH		Ac
4	OSi Me_2t-Bu	H	Ac

Scheme 2a



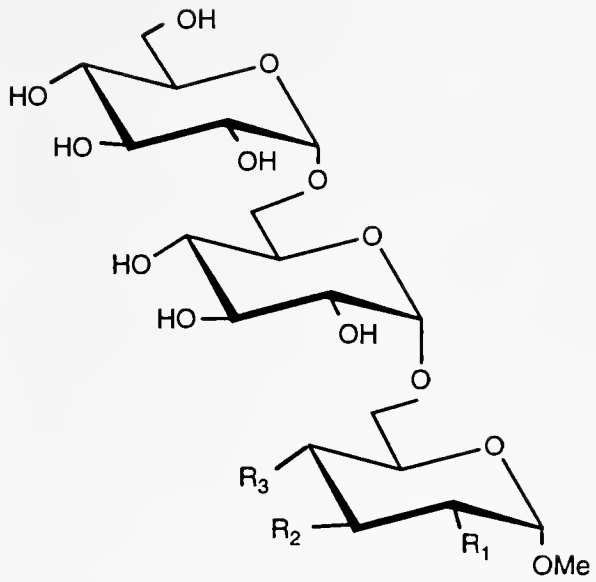
LMC	R_1	R_2	R_3	R_4	R_5
11	OBn	Bn	H	Bz	Ac
12	OBn	Bn	H	Bz	H
15	H	Bz	H	Bz	Ac
16	H	Bz	OBn	Bn	Ac

Scheme 2b



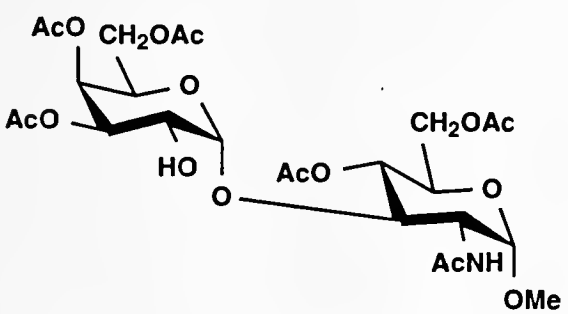
	R_1	R_2	R_3
1 3	H		Bz
1 4	H	OBn	Bn

Study of the Binding Site of Monoclonal, Antidextran Antibody 35.8.2H. In the past we have reported in great detail on the molecular interaction between monoclonal dextran and their antigens when binding to the antigenic terminus only. We are now studying an anti-α(1,6)dextran IgG 35.8.2H, isolated by Kabat and co-workers that is capable of binding internal epitopes of its homologous carbohydrate immunogen. In binding studies we used as ligands methyl α-D-glucopyranoside and methyl α-glycosides of isomalto-oligosaccharides, up to and including the octasaccharide. A fluorescence change was observed with methyl α-isomaltotrioside and higher oligosaccharides, but not with methyl α-D-glucopyranoside or methyl α-isomaltoside. This suggests that the highest affinity subsite is removed from a perturbable tryptophanyl residue. In the methyl α-trioside that does induce a protein fluorescence change, either the glucoside residue or the glucosyl group - respectively the "aglyconic terminus" or the "glyconic terminus" - could occupy the highest affinity subsite. In probing for possible hydrogen bonding we decided to first elucidate the possible contribution to hydrogen bonding of the glucopyranoside residue. Thus, the syntheses of the specifically deoxygenated trisaccharides **1**, **2** and **3** were undertaken. Those trisaccharides specifically deoxygenated at position 2, 3 and 4 (**1-3**) were used for titrations with antibody 35.8.2H. There are only slight changes observed at K_a of 2-deoxygenated trisaccharide (**1**) (Table 2) comparing to methyl α-isomaltotrioside. This could indicate that original hydroxyl at position 2 of methyl α-isomaltotrioside was not involved into the main interactions with protein. On the other hand one can notice that binding constants K_a obtained for trisaccharides **2** and **3** are different comparing to original methyl α-isomaltotrisaccharide. To explain more precisely the reason for the found differences needs additional studies. This experiments are in progress.

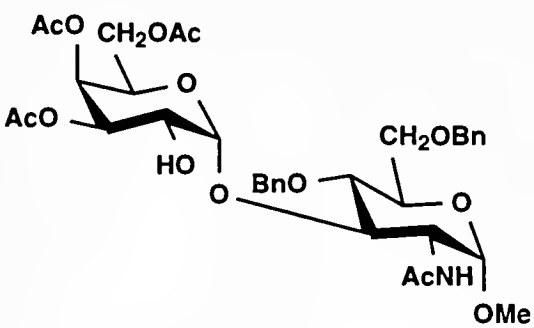


	R_1	R_2	R_3
1	H	OH	OH
2	OH	H	OH
3	OH	OH	H

Oligosaccharides that are Structural Components of the *Sh. Dysenteriae* Type 1 O-Antigen. The critical step in the synthesis of the tetrasaccharide sequence that makes up the repeating unit of *Sh. dysentereae*, namely α-L-Rha-(1,3)-α-L-Rha-(1,2)-α-D-Gal-(1,3)-α-D-GlcNAc is the formation of the α-D-galactosyl-(1,3)-*N*-acetyl-α-D-glucosamine linkage. If the yield and/or stereoselectivity of that reaction could be increased, the overall yield of the tetrasaccharide would improve. Of the several possible approaches, the one described here proposes the construction of a fully protected disaccharide sequence α-D-Gal-(1,3)-α-D-GlcNAc containing a selectively removable protecting group at position 2 of D-galactose, thus offering the possibility to extend the chain at C-2' of the disaccharide. The synthetic strategy described is based on stereoselective α-D-galactosylation at the position 3 of 4,6-O-substituted derivatives of methyl 2-acetamido-2-deoxy-α-D-glucopyranoside. Glycosyl chlorides derived from 3,4,6-tri-*O*-acetyl-2-O-benzyl- and 2-O-(4-methoxybenzyl)-D-galactopyranose have been used as glycosyl donors. Methyl 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-α(3,4,6-tri-O-acetyl-α-D-galactopyranosyl)-α-D-glucopyranoside (**1**) and methyl 2-acetamido-4,6-di-O-benzyl-2-deoxy-3-O-(3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-α-D-glucopyranoside (**2**) have been prepared.



1



2

Mass Spectrometric Structural Studies on Carbohydrate Derivatives. Fully acetylated methyl deoxyfluoro-α-D-glucopyranosides have been studied using electron impact and ammonia chemical

ionization spectrometry. Characteristic differences in the fragmentation of positional isomers were noted on analysis of the spectra. These make it possible to determine the location of fluorine in the molecules studied.

Per-O-acetylated methyl glycosides of D-xylan-type di- and trisaccharides were studied by mass-analyzed ion kinetic energy (MIKE) and collisionally induces dissociation (CID) mass spectrometry using ammonia and methylamine, respectively, as reaction gases in chemical ionization (CI). The oligosaccharides form abundant cluster ions, $[M + NH_4]^+$ or $[M + CH_3NH_3]^+$, and the main fragmentation of these ions in the MIKE and CI spectra is the cleavage of interglycosidic linkages. The spectra allow the determination of the molecular masses of the oligosaccharides, the masses of the constituent monosaccharides, and the branching points in oligosaccharides, if present.

Homo- and heterooligosaccharides composed of hexopyranoses, deoxyhexopyranoses, and acetamidohexopyranoses have been examined by direct chemical ionization mass spectrometry (DCI). Their DCI scan, using ammonia as the reaction gas, gives rise to structurally significant ions. The information thus obtained aids significantly in the sequential analysis of oligosaccharides without derivatization.

Preparation of Glyco-protein Conjugates. Reductive amination is a frequently used technique for the preparation of carbohydrate-based, synthetic antigens, immunoadsorbents and other glycoconjugates. It involves condensation of the carbonyl group of the carbohydrate with the amino group of the carrier to form an intermediate Schiff-base which is chemo-selectively reduced with sodium cyanoborohydride at or near neutral pH. The product is a stable, secondary or tertiary amine. Reductive amination conserves the original net charge on the proteins which is partly responsible for the good retention of their immunogenic activity. The procedure is experimentally undemanding, needs no highly reactive, unstable intermediates and can be applied without difficulty to oligosaccharides containing carboxyl or acetamido groups. Reductive amination has been successfully used for the coupling of free (anomerically unsubstituted) oligosaccharides having D-glucose as the reducing-end, terminal residue. However, this approach could not be applied to oligosaccharides terminated at the reducing end by D-galactose or by a ketose (e. g. fructose or KDO). In such cases reduction of the oxo group was faster than the formation and/or reduction of the intermediate imine . Another disadvantage is that reductive amination of free oligosaccharides converts the reducing-end glycose residue into a polyhydroxy-alkyl fragment. These barriers can be overcome by the use of O- or S-glycosides having an omega-aldehydo(oxa)alkyl aglycon. Regrettably, synthesis of such glycosides is not without difficulty. For example, the aldehydo group of 6-hydroxyhexanal, an apparently ideal candidate as an aglycon, has to be protected to avoid formation of an internal, cyclic hemiacetal. Attempted glycosylation of the derivative 6,6-dimethoxyhexanol under Helferich-conditions leads mostly to methyl instead of the target 6,6-dimethoxy-hexyl glycoside [V. Pozsgay, unpublished results]. Although ozonolysis of the allyl and related glycosides is an established procedure for the preparation of omega-aldehydoalkyl glycosides, it needs special equipment which is not always available. This procedure was shown by NMR spectroscopy to lead to cyclic hemiacetals from allyl and butenyl hexopyranosides. This was independent of the anomeric configuration. The use of such an approach for complex, synthetic oligosaccharides, which has yet to be reported, would put serious constraints on the possibly applicable protecting groups during the synthetic manipulations.

We describe the first preparation of such glycosides by an oxidative procedure other than ozonolysis, by way of examples of a mono- and a disaccharide. Briefly, the procedure involves the synthesis of an 8-hydroxyoctylglycoside (**A**) which is subjected to Swern-oxidation of the C-8 carbon atom of the aglycon to generate the formyl group shown by the general formula **B**, followed by the removal of the protecting groups from the sugar moiety (to give **C**). The C_8 aglycon is similar to Lemieux's hydrophobic spacer. By virtue of the length of the aglycon, the carbon atom of the terminal, formyl group should not be involved in stable, cyclic hemiacetal formation with the sugar hydroxyl groups to any significant extent. Therefore, the proposed spacer moiety was expected to promote efficient and accelerated coupling reactions with matrices having amino groups.

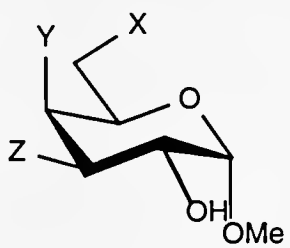
Binding Studies on *Sh. Dysenteriae* Type 1 O-Polysaccharide With Antibody. A recent study permitted the identification of fragment I as an immuno determinant of the O-SP of *Shigella dysenteriae* type 1.
[α-L-Rha-(1,2)-α-D-Gal]
I

To determine the possible role of hydrogen bond interaction of individual hydroxyl groups of that determinant with the monoclonal antibody of reference, deoxy- and deoxy-fluoro analogs of methyl α-D-galactopyranoside and of methyl O-α-L-rhamnopyranosyl-(1,2)-α-D-galacto pyranoside were required.

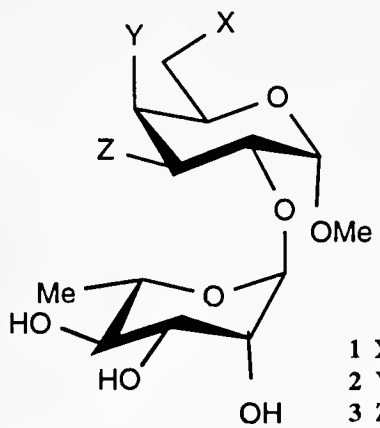
Our strategy for the synthesis of the disaccharides **1**, **2** and **3** involved coupling of the readily accessible tri-*O*-benzoyl-rhamnopyranosyl bromide as the glycosyl donor, with each of the deoxygenated glycosyl acceptors **4**, **5** and **6** under base deficient conditions using trifluoromethane sulphonate as the promoter and sym-collidine as the acid scavenger. Subsequent deprotection of the products of condensation was straightforward.

The nucleophile **4** was prepared from fucose via methyl glycosidation followed by quantitative isopropylidenation. It could be crystallized for the first time and thus, it was fully characterized.

Monodeoxygenation at C-3 and C-4 was performed via Barton's deoxygenation of appropriate (imidazol-1-ylthiocarbonyl) precursors. Particular attention was paid to the choice of the protective groups as well as to the configuration of the starting materials. Two conclusions could be drawn. First: when axial positions are activated *via* the imidazol-1-yl-thiocarbonyl function, radical deoxygenation can be performed more selectively than in the case of their equatorial counterparts. Second: since benzyl groups, especially when at primary position, are somewhat prone to undergo cleavage during the deoxygenation step, the use of substrates protected with the less labile benzoyl groups is preferred for this type of conversion. In view of this, although the nucleophile monodeoxygenated at C-4 could be obtained from derivatives having either the D-galacto or the D-gluco configuration, the pathway involving the former was found superior (scheme 1). Treatment of **7** with 2,2-dimethoxypropane followed by benzylation of OH-2 and subsequent acid hydrolysis gave **8** (65% from **7**). Selective di-*O*-benzoylation of **8** produced the alcohol **9** (72%) which was treated with N,N'-thiocarbonyldiimidazole in refluxing tetrahydrofuran to give the axial substitution product **10** (96%) exclusively. When the reaction was run in other solvents, acyl migration occurred and a mixture of isomers was obtained. Compound **10** was then smoothly deoxygenated to **11** (94%), hydrogenolysis of which afforded the desired methyl 3,6-di-O-benzoyl-4-deoxy-α-D-xylo-hexopyranoside **5**.

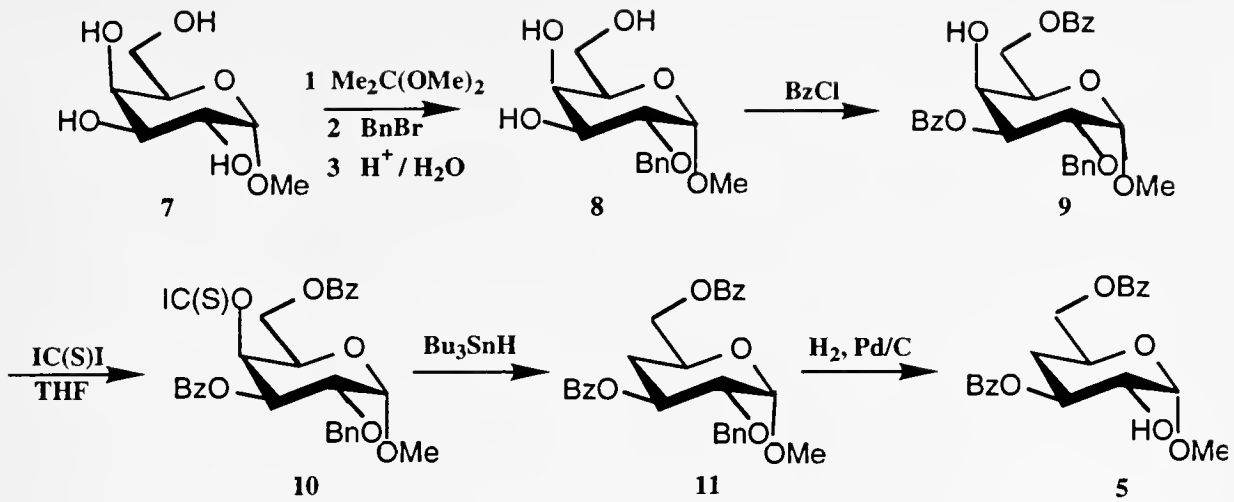


26 X = H Y = OH Z = OH
27 Y = H X = OH Z = OH
15 Z = H X = OH Y = OH
25 X = F Y = OH Z = OH
4 X = H Y, Z = $OC(Me_2)O$



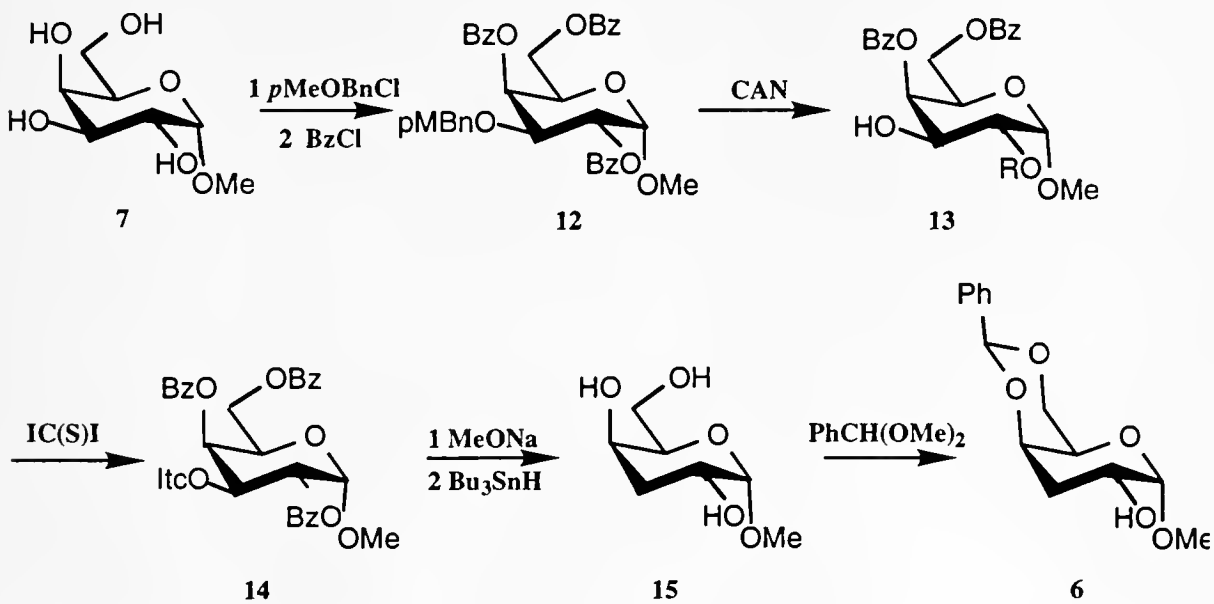
1 X = H Y = OH Z = OH
2 Y = H X = OH Z = OH
3 Z = H X = OH Y = OH

Scheme 1:



Satisfactory conversion of the readily available methyl 2,3,6-tri-O-benzoyl-α-D-galactopyranoside into **14** could not be achieved either in a one step procedure or by a two steps procedure involving first acyl migration then activation with N,N'-thiocarbonyldiimidazole. Therefore, **6** was prepared from **7** as described in scheme 2. Selective protection at position 3 of **7** via stannylation and subsequent benzoylation afforded **12** which was submitted to oxidative deprotection using ceric ammonium nitrate to give **13**, which was converted into **14** upon treatment with N,N'-thiocarbonyldiimidazole. Barton's deoxygenation of **14** followed by deprotection under Zemplen conditions gave the free monosaccharide **15**. To obtain the glycosyl acceptor deoxygenated at position 3 (**6**), compound **15** was treated with benzaldehyde dimethyl acetal.

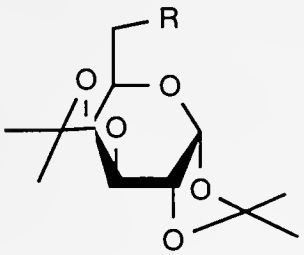
Scheme 2:



Itc = Imidazol-1-ylthiocarbonyl

The 3,4-O-isopropylidene **16** derivative seemed a convenient nucleophile in the synthesis of methyl α-L-rhamnopyranosyl-(1,2)-deoxy-6-fluoro-α-D-galactopyranoside. Therefore, the synthesis of **16** was considered first. Due to the presence of the axial hydroxyl group at C-4, it is known that the displacement of 6-sulphonates and the fluorination of derivatives of D-galactopyranose

is rather difficult. Syntheses of **4** reported to date are based on the further conversion of products of the displacement of a sulphonyl function at C-6 of 1,2:3,4-di-O-isopropylidene-α-D-galactopyranose (**17**).

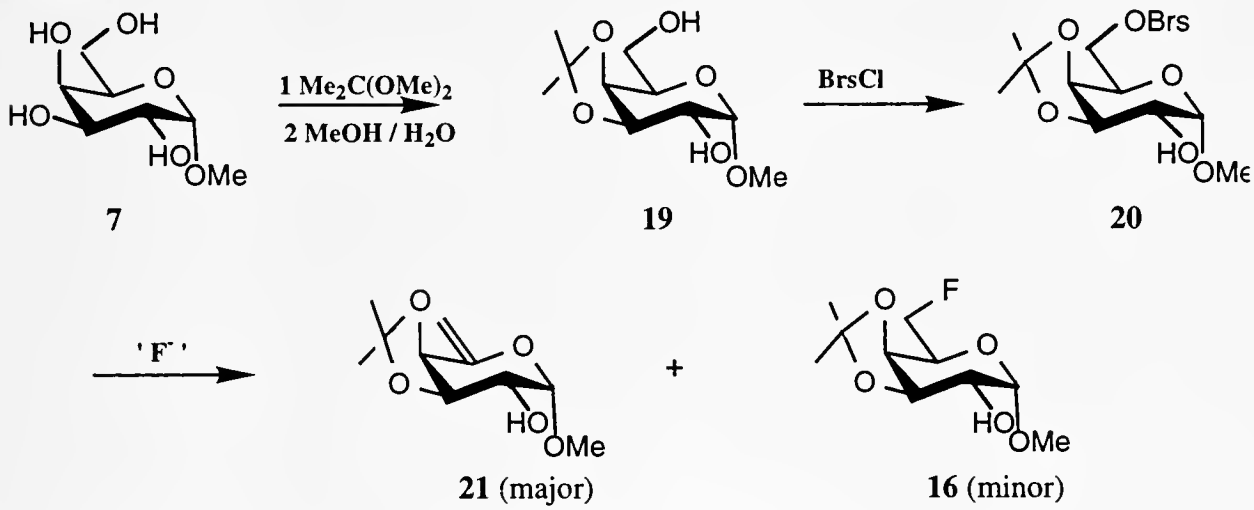


17 R = OH
18 R = F

In a typical example such approach gave **25** in a low yield of 23% from the 6-fluoro derivative **18**. With the aim to improve the accessibility to **25**, we have focused our strategy starting with the commercially available **7**. Since the low yielding, direct fluorination of **7** using DAST was poorly selective and similar treatment of 3,4-O-isopropylidene derivative **19** was still unsatisfactory, a two-step process was considered.

The formation of methyl 3,6-anhydro-α-D-galactopyranoside during nucleophilic displacement of the corresponding 6-sulphonate precursor has been documented. To decrease the nucleophilicity of O-3 in compound **7**, protection at this position was necessary (scheme 3). Therefore, compound **19** was converted to its 6-O-brosyl analog (**20**). Compound **20** was treated with various fluoride donors. The results show that, with this substrate, elimination is favored over the displacement.

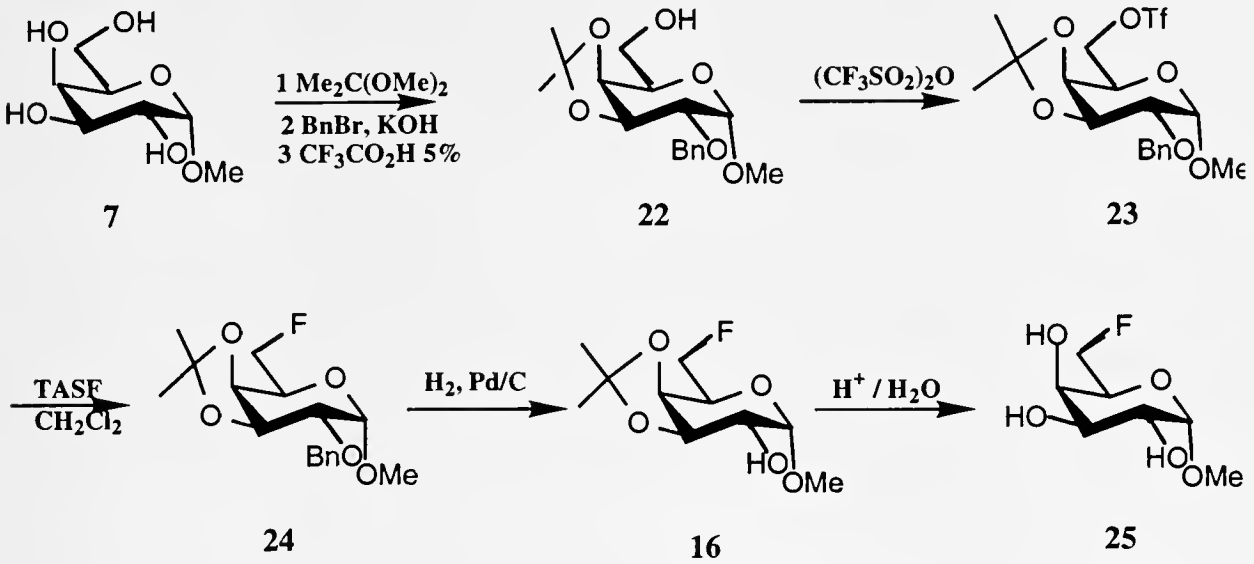
Scheme 3:



Among important side-reactions observed during these experiments was the occasional solvent participation in the displacement reaction and the frequent formation of the exomethylenic derivative (**21**). This occurred under conditions close to those which previously led to satisfactory fluorination of 6-sulphonyl derivatives of **17**. The successful substitution reactions in these situations suggest that in the particular conformation derivatives of **17** adopt, owing to the presence of the 1,2-O-acetal arrangement, the position of the C-O bond at C-4 is altered. On the other hand, satisfactory fluorination at the primary position of C-2 substituted α-D-galactopyranosides were reported. Therefore, we studied the fluorination at position 6 of the 2-*O*-benzyl derivative **22** (scheme 4). Reaction of **22** with DAST was complex. To minimize the side-reactions, the more reactive trifluoromethanesulphonyl ester **23** was prepared and treated with tris(dimethylamino) sulfur(trimethylsilyl)difluoride (TAS-F). This proved very satisfactory and **24** was obtained from **22** in a yield of 83%, under conditions where no product was formed when **20** was the starting material. The different behavior towards the fluorination at C-6 of **20** bearing a free hydroxyl group

at C-2 compared to the one of the fully protected **22** results from the different conformation these two substances adopt as was clearly manifested by their ^{1}H-NMR data.

Scheme 4:



7 22 23 24 16 25

Hydrogenolysis of **24** gave the nucleophile **16** required as a glycosyl acceptor for the preparation of the corresponding disaccharide fluorinated at C-6.

In order to perform the binding studies with the *Shigella dysenteriae* type 1 monoclonal antibodies, the monosaccharides **15**, **26**, **27**, and **26** were also isolated and fully characterized.

1992-1993 Non-project Activity.

Dr. Wayne D. Bowen (Unit on Receptor Biochemistry and Pharmacology, Drug Design and Synthesis Section, LMC) was appointed Adjunct Associate Professor of Biochemistry at Brown University, Providence, Rhode Island. This was a conversion from his tenured Associate Professor position which he vacated upon joining NIH. He continues to serve on doctoral thesis committees of Mitzi Hemstreet (Department of Psychology, Brown University) and Bryan Hoffman (Department of Biochemistry, Brown University). He served on the doctoral thesis committee of Serena Dudek (Department of Neuroscience, Brown University), who successfully completed her degree requirements in 1992. During the reporting period, Dr. Bowen has been invited to present numerous research lectures, including the Second Kelvin Conference in Glasgow, Scotland. He has served as ad hoc reviewer for several biochemical and pharmacological journals.

Dr. John R. Glowa serves as a member of the Editorial Board of the Journal of Neurobehavioral Toxicology and Teratology. He is on the EPA Standing Committee for Risk Assessment for Neurotoxicology. Dr. Glowa is Adjunct Associate Professor of Psychiatry, Department of Psychiatry, USUHS, Bethesda, MD. He is Psychologist in Residence, American University, and Adjunct Associate Professor in Psychology, University of Maryland, College Park, MD. He serves as a reviewer in over ten journals in the area of Behavioral Pharmacology.

Dr. Arthur E. Jacobson (Deputy Chief, LMC) was reappointed as Biological Coordinator of the Drug Evaluation Committee of the College on Problems of Drug Dependence (CPDD) for 1992-1993, and remains Affiliate Professor in the Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University. He is a member of the Editorial Board of the journal *Drug and Alcohol Dependence*. He continues to serve on the selection committee for the International Sato Memorial Award, and is on the Research Evaluation Panel for the Walter Reed Army Institute of Research. Dr. Jacobson is a Charter Fellow of the CPDD, and serves as a reviewer for several Organic and Medicinal Chemistry journals.

Dr. Kenner C. Rice (Chief, LMC) continues as a member of the Editorial Advisory Board of the *Journal of Medicinal Chemistry* and is a Charter Fellow of the College on Problems of Drug Dependence (CPDD). Dr. Rice served as a member of Peer Review Panel C for the Walter Reed Army Institute of Research Promotion Board. He has been appointed to the Advisory Board of *Medicinal Chemistry Research.* During the reporting period, Dr. Rice presented the Glaxo Lecture at the University of Kansas and a lecture at the 34th Buffalo Medicinal Chemistry Symposium in addition to a number of other invited research lectures. Dr. Rice was reappointed as Adjunct Professor of Pharmacology in the Department of Pharmacology and Experimental Therapy, School of Medicine, UMBC. He continues to serve on the selection committee for national and international research awards and is a reviewer for more than 10 scientific Journals.

Patent Applications. In the Drug Design and Synthesis Section two patents were granted this year and 12 other patents are pending. They include (a) Nitrogen-containing cycloheterocycloalkyl aminoaryl derivatives for CNS disorders; (b) N-(arylethyl)-N-alkyl-2-(1-pyrrolidinyl) ethylamines, a novel class of neuroprotective sigma receptor ligands; (c) radiolabeled N-substituted-6-iodo-3,14-dihydroxy-4,5-α-epoxymorphinans, intermediates for producing the same, and a process for the preparation and methods of detecting opioid receptors; and, (d) (+)-isomers of endoetheno/endoethano-epoxymorphinan derivatives as antitussive agents.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 DK 59,501-07 LMC

PERIOD COVERED: October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Design and Synthesis of Drugs Acting on Central and Peripheral Tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: K. C. Rice — Chief, Drug Design & Synth. Section — LMC-NIDDK

Co PI: A. E. Jacobson — Deputy Chief, LMC — LMC-NIDDK

Others: C. Bertha, P. Fleming, J. Pinto, IRTA; B.R. de Costa, Visiting Associate; Z. Gu, NRC Fellow; S. Calderon, S. He, D. Matecka, D. Tadic, Visiting Fellows; S. Kodato, SV. All LMC, NIDDK.

COOPERATING UNITS (if any)
LN-NIDDK (P. Skolnick); U Arizona (F. Porreca); U of Michigan (J. Woods, C.P. France); CC-NM (D. Kiesewetter, M. Channing); U Alabama (E. Blalock); BPB-NIMH (A. Pert); Research Triangle Institute (F.I. Carroll); ARC, NIDA (J.L. Katz, R.B. Rothman, H.C. Akunne); NCI (T.R. Burke Jr.).

LAB/BRANCH: Laboratory of Medicinal Chemistry

SECTION: Drug Design and Synthesis Section

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 6.7	PROFESSIONAL: 6.7	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Synthetic programs are continuing to develop new ligands for imaging brain drug receptors by positron emission tomography (PET) and single photon emission computed tomography (SPECT) scanning, and the NIH Opiate Total Synthesis is employed to provide previously inaccessible unnatural enantiomers of opiates and derivatives. The binding characteristics, pharmacology, immunochemistry, and the multiplicity of opioid receptors were examined, and new drugs were explored as treatment agents for cocaine abuse and for their interaction with the dopamine transporter.

Multiple delta opioid receptors - Nonpeptide opioid receptor ligands with a remarkable 2000 fold binding selectivity for the delta vs mu subtype were synthesized. These compounds are being evaluated in vivo and in vitro. Second generation ligands, which are expected to be extremely valuable probes for further elucidation of the function of the delta receptor subtypes, are being synthesized as affinity labels, tritiated probes and as agents for PET and SPECT imaging of brain delta receptor subtypes in living animals and humans. Such studies will include quantitation of density (Bmax) and affinity (Kd) of this receptor subpopulation in normal subjects and subjects with various disorders involving dysfunction of the opioid receptor endorphin system.

Immunoregulatory opioids - Our studies indicate that opioid-induced immunosuppression is mediated primarily through central mu receptors. DAGO (60 nmol) had no effect on plasma corticosterone or ACTH levels, suggesting that central mu binding does not reduce NK activity through activation of the hypothalamic-pituitary-adrenal-axis.

Potential agents for treatment and prevention of cocaine abuse - Ligand binding and pharmacological studies have revealed two different binding sites for dopamine (DA) uptake inhibitors in the rat caudate nucleus. Synthetic studies have continued toward identification of drugs which will be useful in the treatment and prevention of cocaine abuse are continuing.

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 DK 59,502-05 LMC

PERIOD COVERED: October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Design, Synthesis and Evaluation of Medicinal Agents and Research Tools

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A.E. Jacobson	Deputy Chief, LMC	LMC-NIDDK
Co PI:	K.C. Rice	Chief, Drug Design & Synth. Sect.	LMC-NIDDK
Others:	B.R. de Costa	Visiting Associate	LMC-NIDDK
	M. Mattson	Biologist	LMC-NIDDK
	J.T.M. Linders, S. He	Visiting Fellow	LMC-NIDDK
	T. Jones	Sr. Staff Fellow	LMC-NIDDK

COOPERATING UNITS (if any)
Laboratory of Neuroscience, NIDDK (A.S. Basile, I.A. Paul); Naval Research Lab (C. George); MN-NINDS (M.A. Rogawski); PET Dept., NIH Clinical Center D.O. Kiesewetter); LSU Medical Center (D.J.J. Carr); ARC-NIDA (R.B. Rothman); U of Illinois (M. Reith).

LAB/BRANCH: Laboratory of Medicinal Chemistry

SECTION: Drug Design and Synthesis Section

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 3.0	PROFESSIONAL: 2.0	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Two different binding sites and/or receptor systems were studied: A) PCP binding sites which exist in excitatory amino acid controlled ion channels regulated by glutamate receptors of the NMDA type, as well as in the dopamine uptake complex, and B) Sigma receptors.

A) Our initial electrophilic affinity ligand, metaphit, was found to be an important tool for exploring PCP binding sites. We have now designed and synthesized (+)-3-isothiocyanato-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrochloride, a new, selective affinity ligand for the PCP site which displays little or no irreversible in vitro affinity for opioid, benzodiazepine, muscarinic and dopamine receptors It is the most potent known electrophilic affinity ligand for the PCP binding site. Its potency and selectivity should enable it to be a valuable tool for the continued elucidation of the structure and function of the NMDA receptor-associated PCP binding site in the mammalian central nervous system. Structure-activity studies, including computer-assisted molecular modeling (CAMM), on the new affinity ligand and related molecules enabled the rationalization of the inactivity of its (+)-2-isothiocyanate relative. Additionally, we have noted that the fit of the parent moleule to the PCP pharmacophore (using CAMM) is improved when the previously nonconsidered aromatic ring (the B- instead of the A-ring) is used as part of the pharmacophore.

B) Sigma receptors are non-dopaminergic, non-opioid receptors which bind antipsychotic drugs and have been implicated in neural regulation of motor behavior and modulation of transmitter release upon electrical stimulation of smooth muscle preparations. The immunoregulatory properties of (+)-pentazocine and sigma ligands were examined and the collected data suggested functional and biologically relevant sigma receptors on cells of the immune system. Also, 1N-3-[18F]fluoropropyl-4N-2-([3,4-dichlorophenyl]ethyl)piperazine, a high affinity, selective sigma receptor ligand was synthesized in high radiochemical and chemical purity. It should prove useful for the further characterization of the structure and function of the sigma receptor.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 59602-19 LMC

PERIOD COVERED
October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Interferon Induction and Action. The Antiviral Action of Nucleoside Analogues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. F. Torrence	Section Chief	LMC/NIDDK
Others:	S. Khamnei	IRTA	LMC/NIDDK
	W. Xiao	Visiting Fellow	LMC/NIDDK

COOPERATING UNITS (if any)
Dr. R. Silverman, Cancer Biology, Cleveland Clinic Foundation, Cleveland, Ohio

LAB/BRANCH: Laboratory of Medicinal Chemistry

SECTION: Biomedical Chemistry

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 3.0	PROFESSIONAL: 4.0	OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☒ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Interferon-induced enzyme activities such as the 2-5A synthetase, the 2',5'-phosphodiesterase, and the 2-5A-dependent ribonuclease are studied with the goal of understanding their role in the action of interferon, the induction of interferon by dsRNA, and the control of cell growth and differentiation. Analogues of a mediator of interferon action are synthesized in order to define the relationship between oligonucleotide structure and binding to and activation of the 2-5A-dependent endonuclease. The eventual goal is to understand the biological role of the 2-5A system and to explore the potential of exploitation of this system in chemotherapy. Finally, a number of new approaches to pharmacologically active nucleoside analogues are pursued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-DK 59701-20

PERIOD COVERED
October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Reactions and Immunochemistry of Carbohydrates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Cornelis P. J. Glaudemans	Section Chief, SOC	LMC/NIDDK
Others:	R. S. Arrepalli, E. M. Nashed	Visiting Scientist	LMC/NIDDK
	P. Kovac, E. Petrakova	Visiting Associate	LMC/NIDDK
	L. Mulard	Visiting Fellow	LMC/NIDDK
	V. Pozsgay	Visiting Scientist	LDMI, NICHD

COOPERATING UNITS (if any)

J. B. Robbins, NICHD
R. Schneerson, NICHD

LAB/BRANCH: Laboratory of Medicinal Chemistry

SECTION: Section on Carbohydrates

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 7.0	PROFESSIONAL: 7.0	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Section works on the interaction of (complex) carbohydrate determinants with monoclonal antibodies (MAbs). The elucidation of this interaction - in great molecular detail - is important since it pertains to all ligand-protein interactions. Thus, drug-receptor, effector-receptor as well as viral-receptor interactions may be clarified. We are executing:

1. Physico-chemical studies on antibody/antigen systems.
2. The synthesis of ligands for affinity studies.
3. The manipulation of immunoglobulin genes to produce specifically mutated genes expressing altered antibodies.
4. The study of immunodeterminants of bacteria causing significant diseases on a global scale, so as to evaluate procedures for vaccine development.

We are continuing to determine the specific interaction between microbial polysaccharides such as dextran and a number of monoclonal antibodies, and have prepared ligands to probe the fundamental nature of the antibody-antigen association..

We have prepared additional complex fragments of the capsular polysaccharide of *Shigella dysenteriae* type 1 by sophisticated syntheses. These include deoxy derivatives of the determinant Studies are continuing on the binding area of a monoclonal antibodies towards this disease-causing micro-organism. The variable region of the heavy (VH) and the light (VL) chains have been cloned and sequenced, and they have been incorporated in the bacterial expression vector pSW1, connected by a linker coding for a fifteen amino acid peptide. The expressed protein is a covalently linked sFV. It occurs in inclusion bodies. Attempts to solubilize it in an active form have not yet been successful.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 59801-02 LMC

PERIOD COVERED: October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Evaluation of Potential Cocaine Antagonists

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.R. Glowa	Expert, Behavioral Pharmacol. Unit	LMC/NIDDK
Others:	Frank Wojnicki	IRTA	LMC/NIDDK
	Judd Landsberg	summer IRTA	LMC/NIDDK
	Nolana Kabwit	Howard Hughes student	LMC/NIDDK
	India Aranha	MARC student	LMC/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH: Laboratory of Medicinal Chemistry

SECTION: Drug Design and Synthesis Section

INSTITUTE AND LOCATION: NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 2.9	PROFESSIONAL: 2.0	OTHER: 0.9

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Behavioral Pharmacology Unit is engaged in the assessment of: A) pharmacological agents designed to modify the behavioral effects of substances subject to abuse, using monkey self-administration and drug discrimination, and B) the link between susceptibility to drug abuse and activation of the hypothalamic pituitary adrenal (HPA) axis.

A) The primary current effort of the lab is to characterize the effects of GBR 12909HCl and GBR 12935HCl, as well as sigma-receptor ligands, using cocaine self-administration in monkeys. The effects of GBR 12909HCl and GBR 12935HCl were compared. Both agents selectively decreased cocaine-maintained responding within a limited range of doses, while lower doses had no effect and higher doses decreased responding in both components. These results suggest that these GBR analogs can selectively attenuate cocaine self-administration, without affecting alternative behaviors. In several additional studies, we have been unable to maintain drug-seeking behavior with GBR in cocaine-naive monkeys, in contrast to previous reports of maintenance under substitution paradigms where monkeys have a history of self-administration. These results suggest that GBR-based agents may be useful in the development of drugs to treat cocaine abuse.

B) The primary endogenous agents involves in the HPA axis are corticotropin releasing hormone (CRH), adrenocorticotrophic hormone and cortisol. Several studies have been directed at examining the behavioral specificity of the direct effects of CRH on behavior. The behavioral consequences of the central administration of CRH in rhesus monkeys were determined using food-maintained behavior. When CRH was given repeatedly for several days, its behavioral suppressant effects increased. Home cage food intake, body weight, and subsequent responding for food decreased for up to six weeks before returning to normal. These results suggest that sustained elevations in central levels of CRH can result in a sensitization to its anorexigenic effects, an effect that has not been reported in other species. Because hyperaroused clinical states such as depression, anorexia nervosa, and some forms of drug abuse are characterized biochemically by hypercortisolism and elevated CRH in CSF, these anorexigenic effects may corroborate a potential role for CRH in affective disorders were comorbidity with drug abuse is high.

PHS 6040 (Rev. 1/84)

U.S. GOVERNMENT PRINTING OFFICE: 1991 0-864-939

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 59802-01

PERIOD COVERED
October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Elucidation of the Structure and Function of Sigma Receptors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W. D. Bowen	Research Pharmacologist, Receptor Biochemistry & Pharmacology Unit	LMC/NIDDK
Others:	B.J. Vilner	Visiting Scientist	LMC/NIDDK
	J.M. Cutts, C. Torrence-Campbell	IRTA	LMC/NIDDK
	W. Williams, D. Thomassen	Biologist	LMC/NIDDK
	K.K. Hsu	Summer IRTA	LMC/NIDDK

COOPERATING UNITS (if any)
Research Triangle Institute (F.I. Carroll); U. California, Irvine (R. Matsumoto); Brown University (J.M. Walker); NIMH (K.-S. Lee); U. Arizona (F. Porreca).

LAB/BRANCH
Laboratory of Medicinal Chemistry

SECTION
Drug Design and Synthesis

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 5.6	PROFESSIONAL: 3.4	OTHER: 2.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Sigma receptors are saturable, high affinity binding sites for several important classes of psychotropic drugs including typical antipsychotic, antidepressant, anticonvulsant, and psychotomimetic compounds. Sigma sites are located not only in the central nervous system, but also occur in high density in several peripheral tissues, including liver, kidney, intestine, and endocrine tissue, suggesting important functional roles of these receptors. They are likely to contribute to the beneficial and/or side-effect profile of these compounds. Our unit has investigated several aspects of the molecular and cellular pharmacology of sigma receptors.

Identification of a novel subtype of sigma-1 receptor: Sigma receptors exist in a least two major subtypes, sigma-1 and sigma-2. C6 glioma and some other clonal cell lines were found to possess a sigma-1 site (labeled by [3H](+)-pentazocine) which exhibits subtle differences from the sigma-1 site of guinea pig brain. Lower benzomorphan enantioselectivity and higher calcium ion sensitivity distinguish it from sites of guinea pig brain. This suggests heterogeneity of sigma-1 sites, and may have important functional implications (see below).

Sigma receptor-mediated cytotoxicity: Sigma ligands exhibit high affinity for most typical neuroleptic drugs, including haloperidol. Haloperidol, its metabolites, and several other sigma-active neuroleptics were found to alter the morphology and viability of C6 glioma cells in culture. The compounds caused withdrawal of processes, rounding, and ultimately cell death. Neuroleptics and other compounds which lack affinity for sigma sites had no effect on cells. These effects appear to be mediated by a novel subtype of sigma-1 site identified in C6 glioma and other cell lines. The results suggest that sigma receptors, present in high concentrations in brain motor nuclei, might mediate toxic alterations in cells upon chronic exposure to neuroleptics. This might in turn contribute to irreversible neuroleptic-induced motor abnormalities such as tardive dyskinesia. The data also suggest a possible role of sigma receptors in idiopathic neurodegenerative disorders.

Development of novel sigma agonists and antagonists: Activation of sigma-1 receptors inhibits the ability of muscarinic agonists to stimulate phosphoinositide turnover in brain. Sigma inhibition of the muscarinic (oxotremorine-M) phosphoinositide response was used as a functional assay to screen novel compounds. Despite high sigma binding affinity, several novel ethylenediamines, piperazines, and polyamines exhibited low efficacy, suggesting that they were antagonists or partial agonists. BD1139, a partial agonist, was shown to attenuate the ability of (+)-pentazocine to inhibit oxotremorine-M stimulation. Two other compounds, BD1047 and BD1063 were identified and are being investigated as possible full sigma-1 antagonists. These compounds will be extremely useful for functional studies of sigma sites and have potential therapeutic value.

U.S. GOVERNMENT PRINTING OFFICE 1991 0-554-439

PHOENIX EPIDEMIOLOGY AND CLINICAL RESEARCH BRANCH

Introduction

The Branch conducts epidemiologic and clinical research relating to the origin, development and natural history of non-insulin dependent diabetes and its complications, and obesity, particularly among the Pima Indian population of Arizona. The Branch is based in Phoenix, Arizona and the facilities include a field clinic located in the Hu Hu Kam Memorial Hospital at Sacaton, Arizona on the Gila River Indian Reservation and the clinical research center in the Phoenix Indian Medical Center in Phoenix, Arizona. The Branch consists of three sections, the Clinical Diabetes and Nutrition Section, which conducts clinical research and laboratory investigations; the Diabetes and Arthritis Epidemiology Section, which is involved primarily with the epidemiologic and genetic studies in the Pima Indian population residing on the Gila River Indian Reservation; and the Biometry and Data Management Section which provides data management and support services for the Branch. The Branch serves as a WHO Collaborating Center in Diabetes assisting other units and collaborating centers in methodology, design and analysis of epidemiological and clinical research studies in non-insulin dependent diabetes mellitus.

Summary of Activities

The longitudinal study of the development of diabetes conducted by the Clinical Diabetes and Nutrition Section (CDNS) has previously shown that insulin resistance is a major determinant of the development of diabetes and that a lower insulin secretory response to a bolus intravenous injection of glucose is an additional risk factor for the development of NIDDM in insulin resistant subjects. The molecular and biochemical basis of insulin resistance has formed an important part of the work of the section during the year. Reduced protein phosphatase-1 activity is associated with insulin resistance and this enzyme represents a potential candidate protein. Protein phosphatase-1 is present in muscle in three isoforms and studies using the technique of single stranded conformation polymorphism (SSCP) analysis are being used to screen for mutations in the coding region of these enzymes. Insulin resistant subjects also demonstrate reduced insulin mediated supression of tyrosine phosphatase activity in skeletal muscle. A novel protein tyrosine phosphatase was identified and studies are underway to determine the role of this particular enzyme in insulin resistance.

As insulin resistance is a major risk factor of the development of NIDDM and because insulin resistance is strongly familial, genetic linkage studies have been undertaken to search for relationships with a variety of candidate genes as well as anonymous DNA markers. Linkage between insulin resistance and a region on chromosome 4q, which contains the gene for the intestinal fatty acid binding protein 2 was found in Pima Indians. The exons of the fatty acid binding protein gene have been screened by SSCP

to identify possible mutations. A base transition at position 54 in the 3rd exon of the protein has been identified. Currently studies are underway to determine if this amino acid substitution alters its function. In addition, many other markers, particularly markers on chromosome 11 and chromosome 19 have been screened for genetic linkage with insulin resistance and diabetes. So far no definite evidence of linkage with markers on these chromosomes has been found. Many other anynomous markers on other chromosomes are now being screened.

As a lower insulin secretory response was identified as a further risk factor for NIDDM in insulin resistant subjects and as this is also a familial characteristic, a search for genes linked to the acute insulin response has been performed using sibpair analysis with various candidate genes. Screening for mutations in the glut2 gene, which showed suggestive evidence of linkage, led to the discovery of an amino acid mutation at position 110. This mutation appears to be present in 5-10% of the Pima Indian population. Whether it is associated with differences in glucose transport into islets is currently being investigated.

Obesity is a major with factor for the development of NIDDM and is extremely prevalent among the Pima Indian population. Factors predisposing to the development of obesity in the population have been investigated and several characteristics which predict weight gain have been identified. Studies using the doubly-labelled water method to investigate free-living physical activity in Pima Indian and Caucasian children suggest that the Pima children are less physically active than age-matched Caucasian counterparts. The role of the sympathetic nervous system in determining energy expenditure in under investigation as a determinant of obesity. Early findings indicate that Pima Indians have decreased activation of the sympathetic nervous system compared to Caucasians. The familial aggregation of obesity is also being pursued. Search for genetic linkage with anonymous as well as candidate loci such as the glucocorticoid receptor region of chromosome 5q, the lipoprotein lipase gene on chromosome and the alpha 1 tumor necrosis factor gene on chromosome 6 are being performed.

The Diabetes and Arthritis Epidemiology Section (DAES) continues to perform longitudinal studies of genetic and environmental risk factors for NIDDM and its vascular complications in the Gila River Indian Community. Along with previously described risk factors for NIDDM such as obesity and physical inactivity, low birth weight has been found to be a strong risk factor for the development of diabetes before 40 years of age. Although low birth weight can account directly only for minority of the diabetes in the population, we have proposed that selective survival in infancy of low birth weight infants genetically predisposed to insulin resistance and diabetes can offer an explanation for the observed relationship, and might provide a mechanism of genetic selection which over the course of many generations and under circumstances of nutritional hardship, could

lead to a high population frequency of genetic susceptibility to the disease.

Further investigations of familial aggregation of non-insulin dependent diabetes in the Pima Indian population have shown that the risk of developing diabetes in adolescents and young adults is much greater among offspring who have a parent with diabetes and renal disease than in those who have diabetes without renal disease. These results are compatible with the hypothesis that multiple genetic loci determine the severity and age of onset of diabetes and thus increase the likelihood for development of renal disease among offspring of diabetic parents with renal disease as has been demonstrated previously among the Pima Indians. In conjunction with the CDNS and other collaborators, studies of genetic linkage of highly informative random genetic markers are being conducted to try to identify the location of major genes that determine susceptibility to diabetes.

The Diabetes and Arthritis Epidemiology Section has also investigated the role of weight fluctuation as a potential risk factor for diabetes and examined the relative merits of two widely used screening tests for gestational diabetes. An excess of diabetes among nulliporous women was found and we postulate that this may be the result of high levels of insulin resistance, which are associated with excessive degrees of obesity hyperandrogenemia and decreased fertility. The excess of diabetes found in such women, however, could not be accounted for by the extent of the obesity alone. The section has also continued to conduct epidemiologic studies in arthritis in conjunction with investigators from the National Institute of Arthritis, Musculoskeletal and Skin Diseases. The above activities are described in more detail in the section reports which follow.

Awards, Invited Lectures and Other Activities

Peter H. Bennett, M.B., F.R.C.P., F.F.C.M.
Dr. Bennett was the recipient of the Harry Feldman Award of the American Epidemiological Society and delivered the Harry Feldman Lecture at the Annual meeting of the American Epidemiological Society in Pittsburgh, PA on March 25, 1993.

Dr. Bennett also received the Superior Service Award of the Department of Health and Human Services in 1993.

Dr. Bennett continues to serve on the editorial boards of Diabetes and Metabolism Reviews, Acta Diabetologica and as Associate Editor of the American Journal of Epidemiology.

Foreign Activities

Dr. Bennett served as a temporary advisor for the World Health Organization (WHO) and as an invited speaker at the 1st Eastern Mediterrean Regional Diabetes meeting held in Karachi, Pakistan in December 1992. He served as a temporary advisor to WHO in Kuwait

in investigating and making recommendations concerning the extent of diabetes in that nation and on the design and implementation of a national diabetes control program. In July 1993, he served as a member of the full-time faculty for the joint WHO-International Diabetes Federation international seminar on the Public Health Aspects of Diabetes Mellitus in Cambridge, England. Dr. Bennett was also an invited speaker and temporary advisor to WHO at the International Clinical Epidemiology in Diabetes Workshop held in Omiya, Japan.

Dr. Bennett served as a member of the WHO Study Group on the Prevention of Diabetes Mellitus held in Geneva, Switzerland in November 1992.

Clinical Diabetes and Nutrition Section

The scientific mission of the section is to determine the etiology and pathogenesis of the non-insulin dependent diabetes mellitus (NIDDM) as it occurs among the Pima Indians of Arizona. To achieve this goal the major effort of our section has been to conduct a cross-sectional and prospective study of non-diabetic Pima Indians to determine the metabolic factors that predict the subsequent development of the disease. Beginning in 1982, we entered over 300 subjects in to this large study in which Indian volunteers are admitted to the clinical research ward for approximately 10 days and undergo studies of body composition, insulin secretion, and insulin sensitivity. In the past decade over 40 subjects have developed NIDDM during follow-up. Analysis of the data to date show that obesity and insulin resistance are major risk factors for the development of NIDDM. In addition, among insulin resistant subjects, a lower insulin secretory response to an intravenous bolus injection of glucose is a minor risk factor for the subsequent development of NIDDM. The other major metabolic characteristic of NIDDM, over production of glucose by the liver, was not a prediabetic abnormality, and therefore, develops secondarily in the natural history of the disease. Because insulin resistance, reduced insulin secretory response to glucose, and obesity have each been identified as prediabetic abnormalities the section has focused increased effort to determine the underlying causes of these abnormalities in the Pimas.

Insulin Resistance. Since insulin resistance is a major risk factor for the development of NIDDM in the population, we have undertaken a series of physiologic experiments, combinations of in vivo and in vitro studies, and a genetic approaches to understand the causes of insulin resistance in the Pima population.

Recent experimental animal studies, and a preliminary study in man, suggested the possibility that there is a large arterial, interstitial fluid insulin gradient. Studies were undertaken to determine whether this gradient was greater in insulin resistant subjects than insulin sensitive subjects. A technique was

developed to measure arterial and interstitial fluid insulin concentrations simultaneously during insulin infusion. The studies of insulin infusions in both lean, insulin sensitive individuals, and insulin resistant subjects demonstrated that there is a larger arterial/interstitial fluid gradient, but this gradient is similar in insulin resistant and insulin sensitive subjects such that the major cause of the insulin resistance appears to be in the target tissue itself.

Previous in vivo experiments by our group and others demonstrated that the insulin resistance in man is largely accounted for by reduced insulin mediated glucose uptake in skeletal muscle. In the past, we have shown that there is a good correlation between insulin mediated glucose uptake into the whole body and insulin stimulation of skeletal muscle glycogen synthase. The reduced insulin stimulated glycogen synthase activity of insulin resistant subjects was subsequently also shown to be associated with a reduced basal and insulin stimulated protein phosphatase-1 activity, which is the enzyme that dephosphatases and activates glycogen synthase. We have continued studies of this enzyme as it remains an important candidate protein for explaining the insulin resistance leading the high prevalence of diabetes among the PImas.

Skeletal muscle tissue from insulin sensitive and insulin resistant subjects have been obtained for further studies of protein phosphatase (PP-1) activity. These muscle samples have been studied before and after insulin treatment, before and after exposure to manganese, and before and after exposure to azide. The results of these studies suggest that azide reverses the effect of an inhibitor of manganese activation, which has been localized to the glycogen - microsomal fraction of the insulin sensitive subject and that has decreased effects in insulin resistant subjects. In addition, insulin resistant subjects appear to have increased binding of their PP-1 gamma isoform of the PP-1 enzyme to the glycogen-microsomal fraction. Taken together, the results to date suggest that the abnormal PP-1 activity in insulin resistant subjects may indirectly involve the interaction of PP-1 with the G-subunit, which is located within this glycogen microsomal subfraction. Studies are currently underway to define the genomic structure of the three isoforms of the PP-1 which is now known to exist in skeletal muscle. This will allow us, using single stranded conformation polymorphism analysis (SSCP), to screen for mutations in the coding regions of these enzymes in the insulin resistant subjects.

In addition to studying the theorine/serine protein phosphatase-1, studies have also been conducted of tyrosine phosphatase activity (PTPase) in skeletal muscle of insulin resistant and insulin sensitive subjects. Since we have previously demonstrated a reduced insulin mediated suppression of tyrosine phosphatase activity in the skeletal muscle of insulin resistant Pima Indians. A novel protein tyrosine phosphatase was identified in human skeletal muscle using the polymerase chain reaction and

degenerate primers. This PTPase contains several PEST sequences and is being expressed in SF9 cells using a recombinant baculovirus system. Studies are underway to determine the role of this particular PTPase in insulin action in insulin sensitive and insulin resistant Pimas. In addition, SH2-domain PTPases were sought in FAO cells, a rat insulin-sensitive, hepatoma cell line and in C2C12 cells, a mouse muscle cell line, using RT-PCR and degenerate primers. Both lines were found to express insulin-sensitive SH2 containing PTPases and these are being fully characterized in these insulin responsive cell lines.

We have also previously observed that insulin resistance for glucose uptake is associated with decreased action of insulin on the regulation of gene expression for several genes. In particular, C-HA-RAS and MYF5 showed rapid changes in messenger RNA levels in skeletal muscle during an insulin infusion. In insulin resistant subjects the changes in these RNA levels of these two genes were greatly reduced compared to insulin sensitive subjects. To understand the mechanism of insulin resistance of gene expression, the CIS- acting sequences and transacting factors that bind these sequences and are responsible for the changes in RNA levels for these two genes during insulin stimulation are being investigated. Studies will then be undertaken using gel shift assays, to determine the abundance of transacting factors in insulin resistant and insulin sensitive individuals under the hypothesis that insulin resistant individuals may have reduced levels of these transacting factors.

Since insulin resistance is a major risk factor for development of NIDDM in Pima Indians, and because insulin resistance is strongly familial characteristic in the population, we have also undertaken studies to look for genetic linkage between insulin resistance and a variety of candidate genes as well as anonymous DNA markers. In particular, we observed linkage between a region on chromosome 4q, containing the gene for the interstitial fatty acid binding protein and the gene for annexin V protein, and insulin action in Pimas. The exons of the liver fatty acid binding protein gene were screened using SSCP to identify potential mutations. A base transition that resulted in an amino acid change from alanine to threonine at position 54 in the protein was identified. Whether this amino acid substitution in the protein alters the function of the protein is currently under investigation.

We have also screened many other markers for linkage with insulin resistance, including markers on Chromosome 11 which includes the insulin gene, as well as markers on Chromosome 19 which includes the insulin receptor gene as well as the glycogen synthase gene. Sib-pair linkage analysis have not indicated definite linkage between any of these markers and insulin resistance. Many other anonymous markers are now being screened for potential linkage within insulin resistance. These markers are being chosen for being highly polymorphic and for being uniformly distributed across the genome.

Insulin Secretion

A lower insulin secretory response to a intravenous bolus injection of glucose was identified as an additional risk factor for the development of NIDDM in insulin resistant Pima Indians. The acute insulin response has also been found to be a highly familial characteristic and the frequency distribution of the acute insulin responses in the Pima population with normal glucose tolerance is significantly different from a single normal distribution. These data suggest that a lower acute insulin response to glucose is not only a prediabetic abnormality, but that it may also have genetic determinants.

We began to search for possible genes determining the acute insulin response by performing sib-pair linkage analysis with various candidate genes. Initial studies indicated no linkage between the glucokinase locus (chromosome 7) and the acute insulin response. We could not exclude, however, linkage between the liver/islet glucose transporter (glut2) locus and the acute insulin response. Therefore, the GLUT2 gene was screened for mutations in its exons by using SSCP. An abnormal migration pattern of exon3 in several individuals was identified. On sequencing this turned out to be due to a base transition resulting in a change in the amino acid at position 110 in the protein from a theorine to a isolucine. This amino acid change appears to occur in approximately 5-10% of the Pima population. Whether this amino acid substitution in the protein is associated with differences in glucose transport into islets is currently under investigation.

In addition to looking for linkage between candidate genes and the acute insulin response, we have also looked for linkage between several different anonymous markers, all of which were used in the linkage analysis with insulin action. No linkage between these anonymous markers and the acute insulin response have been found, but there are very large areas of the genome that are yet to be screened for linkage.

Obesity

Obesity is extremely prevalent among the Pima Indians and is a major risk factor for the development of NIDDM. For this reason, we have focused many of our physiologic studies, and more recently our genetic studies to determine factors that may predispose the development of obesity in the population. Four metabolic parameters have been found to be familial and also to predict weight gain in the population. These are: the resting and 24h sedentary metabolic rate, 24h respiratory quotient, insulin sensitivity, and spontaneous physical activity. The familial aggregation of these four parameters suggested the possible genetic background to energy metabolism and it's components. Studies are also underway to measure the level of free-living physical activity in Pima Indian and Caucasian children. Preliminary analyses of the data suggest that the Pima Indian children are less physically active than their age-matched Caucasian counter-parts. It is

likely that this is an important contributing factor to the higher prevalence of obesity among the Pimas, but prospective studies to demonstrate this directly are required. The reason for the reduced physical among the Pima children, however, is not clear and may be to "inherent" causes.

We have previously demonstrated the sympathetic nervous system activity is reduced in Pimas compared to Caucasians and more recent studies have indicated that in addition to a decrease basal activity, the Pimas have a decreased activation of the sympathetic nervous system following ingestion of glucose. It is possible this is due to a decrease response of the sympathetic nervous system to insulin; another potential expression of an obesity/insulin resistance syndrome. In addition to examining the role of energy expenditure in determining obesity, studies have also been conducted to assess the role of variations in food intake in causing obesity using a new developed automated food selection system on our clinical research ward. Studies using this system have demonstrated that most subjects tend to overeat given free access to food using this system and this overfeeding is mainly due to fat imbalance rather than imbalance of carbohydrate or protein. Surprisingly, the rate of food intake correlated inversely with the degree of obesity indicating that a slower food intake rate may possibly be associated with a reduction in satiety due lack of gastric distension and that this may be a contributing factor to the development of obesity.

In conjunction with our genetic linkage studies with insulin resistance and insulin sensitivity, we also have looked for linkage between a variety of candidate loci and obesity in the population. We have previously observed suggestive evidence of association between the ABO locus on chromosome 9 and the body mass index, an estimate of obesity. A large of number of subjects are therefore being typed at a highly polymorphic, anonymous marker nearby the ABO locus to search for linkage between this region of the chromosome and obesity in the Pimas. Other candidate loci being investigated for linkage with obesity are the glucocortoid receptor region on chromosome 5q and the lipoprotein lipase gene on chromosome 8, as well as the alpha-tumor necrosis factor gene on chromosome 6.

Invited Lectures and Invited Participation in Symposia

Clifton Bogardus, M.D.

" Genetics and low rates of energy expenditure in the etiology of obesity in man", 13th Congress of the Japan Society for the Study of Obesity, The 3rd Department of Medicine, Osaka, Japan, October, 1992

"The Etiology of NIDDM in Pima Indians", University of Washington, Seattle, Seattle, Washington, December, 1992

"Metabolic Changes in NIDDM" and "Genetic Studies in Pima Indians", Howard Hughes Medical Institute Research Laboratories, Chateau de Maffliers, France, March, 1993

"Genetics of NIDDM in Pima Indians", Diabetes Research Training Center at the Albert Einstein College of Medicine, New York, New York, April, 1993

"Studies of the Etiology of Insulin Resistance in Pima Indians", University of Vermont, Dept.of Medicine, Burlington, VT, June, 1993

Eric Ravussin, Ph.D.

"Hypometabolism as a Predisposition for Obesity", Institute of Experimental Endocrinology, Bratislava, Czechoslovakia, September, 1992

"Pathophysiology of Obesity in Man: Lessons Learned from the Pima Indians", University Cattolica Del Sacro Cuore, Rome, Italy, December, 1992

"Alcohol and Body Mass Maintenance", National Institute on Alcohol Abuse and Alcoholism, Bethesda, Maryland, January, 1993

"Energy Expenditure and Exercise", The American Institute of Life-Threatening Illness and Loss, New York, New York, April, 1993

"Obesity in the Pima Indians: Genetics vs. Environment", National Science Foundation International Program, Sydney, Australia, May, 1993

"Low fat/carbohydrate oxidation ratio and insulin sensitivity as risk factors for subsequent weight gain", The Royal Veterinary and Agricultural University, Copenhagen, Denmark, May, 1993

Diabetes and Arthritis Epidemiology Section

The Diabetes and Arthritis Epidemiology Section has continued the longitudinal studies of genetic and environmental risk factors for diabetes and its vascular complications in the Pima Indians, as well as continuing epidemiologic studies of arthritis,

cholelithiasis, mortality rates, and causes of death. Birth weight and risk of NIDDM in adults. The prevalence of diabetes was examined at ages 20-39 years in 1179 Pima Indians born between 1940 and 1972 to determine if diabetes is associated with birth weight. The prevalence of diabetes was greatest among those with the lowest and among those whith the highest birth weights, although most of the diabetes occurred in subjects who had been of normal birth weight.

Given the high mortality of low birth weight infants, we propose that selective survival in infancy of those genetically predisposed to insulin resistance and diabetes provides an explanation for the observed relationship between low birth weight and diabetes and for the high prevalence of diabetes in many populations.

Familial aggregation of NIDDM and renal complications. The risk of diabetes in adolescence and young adulthood is greater in persons with a parent with diabetes and diabetic renal disease than in those whose parents have diabetes without renal disease. The results are compatible with the hypothesis that multiple loci (or homozygosity at a single locus) determine the severity of diabetes and the likelihood of developing renal disease.

Weight fluctuation, risk of NIDDM, and mortality rates. Although the degree of overweight (or body weight relative to height) is a strong predictor of diabetes in the Pimas, the degree of weight fluctuation (or departure from a linear change in weight over time) was not associated with risk of diabetes or with increased mortality rates, except from trauma in men. Thus, concern about weight fluctuation should not deter clinicians from advising weight loss for diabetes prevention or other health benefits.

Genetics of obesity. Studies of the familial pattern of obesity suggest the influence of a major gene, but with additional influences of recent changes in environmental or behavioral factors. Linkage studies to locate a major gene are underway.

Glucose Intolerance in Pregnancy. World Health Organization (WHO) and National Diabetes Data Group (NDDG) criteria were compared in non-fasting Pima Indian women without a history of diabetes. The WHO criteria, as a screen for gestational diabetes, identified all women with gestational diabetes, and missed fewer perinatal complications than the more cumbersome NDDG procedure.

Diabetes incidence and prevalence in Pima women with no history of pregnancy were compared with the incidence and prevalence in women who had had at least one pregnancy. Those who had never been pregnant had a higher prevalence of non-insulin-dependent diabetes and were more obese than women who had been pregnant. Among women followed to age 40 years, those who had not been pregnant by the baseline examination were at significantly higher risk for developing non-insulin-dependent diabetes. This difference could be accounted for by a higher degree of obesity,

but controlled for age and obesity, nondiabetic women who had never been pregnant had significantly higher fasting plasma glucose concentrations and higher fasting and 2-hour serum insulin concentrations than nondiabetic women who had experienced at least one pregnancy. We hypothesize that some Pima Indian women who have a high risk for non-insulin-dependent diabetes develop obesity and hyperinsulinemia at an early age and may develop an associated hyperandrogenemia and decreased fertility.

The abilities of 2-hour post-load plasma glucose and glycosylated hemoglobin to predict diabetes were examined in pregnant nondiabetic Pima Indian women. Third trimester 2-hour glucose, obesity, age and parity each predicted future diabetes, but third trimester glycosylated hemoglobin did not. Thus, we hypothesize that relatively acute changes in glucose tolerance during the third trimester in women later destined to develop non-insulin-dependent diabetes are reflected by the 2-hour glucose but not the glycosylated hemoglobin.

Arthritis. The section continues epidemiologic research in the epidemiology of rheumatoid arthritis in collaboration with the National Institute of Arthritis and Musculoskeletal Diseases. Pima Indians with rheumatoid arthritis have only a slight increase in age-specific mortality rates, in contrast with reports from clinic series in which the mortality rate is substantially increased. There has been a decreasing trend in the incidence of rheumatoid arthritis during the last 25 years in the Pima population.

Mortality from Gallstone Disease. Mortality rates were determined during a 20-year follow-up of 383 Pima Indians known to have had gallstone disease or to have had normal gallbladder x-rays in 1966 to 1969. Among those with gallstone disease, the all-cause death rate was nearly twice as high, and the death rate due to malignancies was over 6 times as high, as among those without. The higher non-malignancy death rates are unexplained.

Other Activities. Section staff continue to be active in medical research and education beyond the projects described here. Staff collaborate extensively in research projects conducted by the Clinical Diabetes and Nutrition Section, NIDDK. They continue lecturing at universities and contributing to national and international meetings and workshops.

Invited Lectures and Invited Participation in Symposia:

William C. Knowler:

"Familial Factors in Diabetes in the Pima Indians", Symposium on Diabetes in the Aboriginal Populations, University of Manitoba, Winnipeg, Canada, November 20, 1992.

"Diabetic Nephropathy in the Pima Indians", Symposium on Kidney Disease of Diabetes Mellitus, National Institute of Diabetes and Digestive and Kidney Diseases, April 29, 1993.

"Prevention of Type 2 Diabetes", International Diabetes Epidemiology Group, Noumea, New Caledonia, September 21-23, 1993.

David J. Pettitt:

"Malconditioning of Progeny by Maternal GDM: (Experience with Pima Indians)", 2nd International Graz Symposium on Gestational Diabetes. October 12-16, 1992, Graz, Austria.

"Long-term Implications of Gestational Diabetes Mellitus for the Offspring", International Workshop on Adverse Perinatal Outcomes of Gestational Diabetes Mellitus, sponsored by the National Institute of Child Health and Human Development and the National Institute of Diabetes and Digestive and Kidney Diseases. December 3-4, 1992, Bethesda, Maryland.

"Long Term Outcome of Offspring of Gestational and Type II Diabetics", Gallup Indian Medical Center Clinical Staff Conference. January 28, 1993. Gallup, New Mexico.

"Abnormal Glucose Tolerance in Pima Indian Pregnancies: Implications for the Mother and Child", Indian Health Service Navajo Area Joint Obstetrician -Pediatrician Annual Midwinter Meeting. January 30, 1993, Telluride, Colorado.

"Diabetes and Pregnancy in Pima Indians", American College of Obstetricians and Gynecologists / Indian Health Service Consultation Meeting on Diagnosis of Gestational Diabetes. April 12, 1993, Washington, D.C.

Panelist and Discussant: "Pregnancy and Diabetes: Enhancing Quality Care with Partnership Management", Indian Health Service Diabetes Mellitus Program. April 22, 1993, Sacaton, Arizona.

"Long Term Outcome for the Offspring of Gestational and Type II Diabetics", American Diabetes Association Council on Diabetes and Pregnancy Annual Program. June 12, 1993. Las Vegas, Nevada.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69000-28

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Diabetes Mellitus and Other Chronic Diseases in the Gila River Indian

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W.C.	Knowler	Chief	DAES, NIDDK
Others:	P.H.	Bennett	Chief	PECRB,NIDDK
	D.J.	Pettitt	Assistant Chief	DAES, NIDDK
	D.R.	McCance	Visiting Associate	
	R.L.	Hanson	Epidemiology Staff Fellow	
	K.M.V.	Narayan	Visiting Scientist	

COOPERATING UNITS (if any)

Indian Health Service; National Institute of Arthritis and Musculoskeletal and Skin Diseases; Ariz. State U.; Cleveland Clinic, Phoenix, AZ; University of Pittsburgh.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.7	1.7	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this project is to identify the determinants of non-insulin-dependent diabetes (NIDDK), various types of arthritis, and gallbladder disease, and elucidate the natural history of the diseases. Genetic and environmental risk factors for NIDDM have been studied in the Pima Indians. The residents of the study area, approximately 5000 people, have participated in a longitudinal population study since 1965, allowing observations of the natural history of diabetes mellitus. Risk factors for obesity, hypertension, and cholelithiasis are also studied, along with the relationships of these diseases to diabetes and their effects on mortality rates. The genetics of diabetes is studied by means of family studies and relationships of genetic markers to disease. The roles of obesity, serum insulin concentrations, impaired glucose tolerance, occupational and leisure-time physical activity and diabetes in relatives are assessed.

Birth weight was found to be related to subsequent risk of diabetes in adulthood, with those of low (less than 2.5 kg) and high (at least 4.5 kg) birth weights having a higher risk than those of intermediate birth weights. A proposed explanation is selective survival in infancy of those genetically predisposed to insulin resistance and diabetes. The risk of diabetes in adolescence and young adulthood is greater in persons with a parent with diabetes and diabetic renal disease than in those whose parents have diabetes without renal disease. The results are compatible with the hypothesis that multiple loci (or homozygosity at a single locus) determine the severity of diabetes and the likelihood of developing renal disease. Although the degree of overweight (or body weight relative to height) is a strong predictor of diabetes in the Pimas, a study of weight fluctuation (or departure from a linear change in weight over time) was not associated with risk of diabetes. Thus, concern about weight fluctuation should not deter clinicians from advising weight loss for diabetes prevention. Studies of the familial pattern of obesity suggest the influence of a major gene, but with additional influences or recent changes in environmental or behavioral factors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69001-24

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Complications and Outcome of Diabetic and Prediabetic Pregnancies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D.J. Pettitt	Assistant Chief	DAES, NIDDK
Others:	P.H. Bennett	Chief	PECRB, NIDDK
	W.C. Knowler	Chief	DAES, NIDDK
	R.L. Hanson	Epidemiology Staff Fel	DAES, NIDDK
	D.R. McCance	Visiting Associate	DAES, NIDDK
	K.M.V. Narayan	Visiting Scientist	DAES, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Biostatistics and Data Management Section, PECRB;
Mayo Clinic, Rochester, Minnesota (B.A. Kottke)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.4	1.0	0.4

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purposes of the project are to determine the effects of abnormal glucose tolerance on outcome of the pregnancy, to determine long term prognosis for the women and their offspring, and to identify diabetes and impaired glucose tolerance during pregnancy in women in the Gila River Indian Community. By means of a glucose tolerance test as well as chart review, the diabetes status of every woman is determined at two-yearly intervals and during the third trimester of each pregnancy. Pima Indian women were evaluated in cross-sectional and longitudinal analyses. Women who had never been pregnant had a higher prevalence of non-insulin-dependent diabetes and were more obese than women who had been pregnant. Among women followed to age 40 years, those who had not been pregnant by the baseline examination were at significantly higher risk for developing non-insulin-dependent diabetes. This difference could be accounted for by a higher degree of obesity, but controlled for age and obesity, nondiabetic women who had never been pregnant had significantly higher fasting plasma glucose concentrations and higher fasting and 2-hour serum insulin concentrations than nondiabetic women who had experienced at least one pregnancy. We hypothesize that Pima Indian women who have a high risk for non-insulin-dependent diabetes develop obesity and hyperinsulinemia at an early age and may develop an associated hyperandrogenemia and decreased fertility. In another analysis, the abilities of 2-hour post-load plasma glucose and glycosylated hemoglobin to predict diabetes were examined in pregnant nondiabetic Pima Indian women. Third trimester 2-hour glucose, obesity, age and parity each predicted future diabetes, but third trimester glycosylated hemoglobin did not. Thus, we hypothesize that relatively acute changes in glucose tolerance during the third trimester in women later destined to develop non-insulin-dependent diabetes are reflected by the 2-hour glucose but not the glycosylated hemoglobin.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69006-23

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gila River Indian Community Autopsy and Mortality Study

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P.H. Bennett	Chief	PECRB,NIDDK
Others:	W.C. Knowler	Chief	DAES, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	M.L. Sievers	Guest Researcher	DAES, NIDDK
	R.L. Hanson	Epidemiology Staff Fellow	DAES, NIDDK

COOPERATING UNITS (if any)

Pathology Department, Phoenix Indian Medical Center, Indian Health Service, Phoenix, Arizona; Cleveland Clinic Foundation, Phoenix, Arizona;

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The causes of death and postmortem characteristics of Pima Indians of the Gila River Indian Community are investigated so that findings in subjects with and without diabetes mellitus can be correlated with studies in living subjects. Medical records are reviewed for the determination of cause of death and for the occurrence of certain serious diseases or complications of diabetes.

The purpose of the study is to relate the outcome and cause of death to events or risk factors measured in life among Pima Indian residents of the Gila River Indian Community, particularly in relation to diabetes, cardiovascular diseases and gallbladder disease. Death Certificates are obtained on all members of the Gila River Indian Community In addition, post mortem examinations and all available medical records pertaining to the subjects are obtained to ascertain conditions present at the time of death and ascertain cause of death as precisely as possible. These records are reviewed in a standardized way for evidence of the complications of diabetes, vascular disease, neoplasms and other conditions. The records are used to determine the causes of death and incidence of complications associated with diabetes and other conditions identified initially during life by the longitudinal epidemiologic studies in the population.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69009-28

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Natural History of Arthritis and Rheumatism in the Gila River Indian

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P.H. Bennett	Chief	PECRB, NIDDK
Others:	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	W.C. Knowler	Chief	DAES, NIDDK
	S.P. Heyse	Chief	OPECA, NIAMS
	L. Jacobsen	Visiting Associate	OPECA, NIAMS

COOPERATING UNITS (if any)

Indian Health Service, Arizona State University; NIAMS

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.7	0.3	0.4

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The development and progression of osteoarthrosis, rheumatoid arthritis and ankylosing spondylitis are being determined by means of clinical, radiographic and serological examinations carried out prospectively at two-yearly intervals among adults of the Gila River Indian Community (Pima Indians) in Arizona, in conjunction with epidemiological studies of diabetes in the same community. The purpose of this investigation is to ascertain the determinants of these diseases in the population, and to identify factors which predispose to or alter the natural history of progression of the disease. Host factors such as age, sex, and various gene markers including HLA and Gm, together with various potential environmental determinants, such as obesity and evidence of exposure to infectious agents, are being investigated prospectively to determine their relationship to the development of these diseases. Longitudinal data have now been collected for 25 years and represent a unique data set for epidemiological studies of arthritis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69015-11

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cross-Sectional and Longitudinal Study of "pre-diabetes" in the Pima

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus,	Chief	CDNS, NIDDK
Others:	S. Lillioja	Visiting Scientist	CDNS, NIDDK
	M. Spraul	Visiting Associate	CDNS, NIDDK
	D. Mott	Supervisory Res. Chem.	CDNS, NIDDK
	R. Pratley	Senior Staff Fellow	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.05	.85	.20

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Pima Indians of Arizona have the highest reported prevalence and incidence of non-insulin dependent diabetes mellitus (NIDDM) of any population in the world. Since 1982, our section has been studying a subset of this population to determine the etiologic factors that predispose individuals in the population to develop the disease. Subjects are admitted to the clinical research ward on a yearly basis to undergo body composition analysis, an oral glucose tolerance test, an intravenous glucose tolerance tests, a standard mixed meal test, and a two-step hyperinsulinemic, euglycemic clamp to measure insulin action in vivo. Over 300 individuals have been entered into the study and in the past decade approximately 40 subjects have developed NIDDM. Four major metabolic characteristics of subjects with NIDDM have been identified: obesity, insulin resistance, abnormal insulin secretory responses to glucose, and over production of glucose by the liver. Of these four major metabolic characteristics, obesity and insulin resistance appear to be major risk factors for the development of NIDDM. A reduced acute insulin secretory response to a glucose stimulus appears to be an additional, minor risk factor. Over production of glucose by the liver does not appear to be a metabolic abnormality that precedes the development of NIDDM. The over production of glucose by the liver appears to develop later in the natural history of the disease. In the past few years this study has been extended to include an analysis of siblings of individuals originally entered into the study to allow genetic linkage analysis between various candidate genes and anonymous DNA markers and the three pre-diabetic abnormalities-insulin resistance, abnormal insulin secretory responses to glucose, and obesity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69020-09

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin Resistance and the Regulation of Muscle Glycogen Synthase

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	D. Mott	Supervisory Res. Chem.	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Second Department of Medicine, University of Helsinki, Helsinki, Finland (H. Yki-Jarvinen)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.65	.05	.60

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are currently characterizing the abnormalities for regulation of human muscle glycogen synthesis in insulin-resistant subjects. In insulin-resistant subjects, fasting glycogen synthase phosphatase and phosphorylase phosphatase activities are reduced and fail to show the peak insulin stimulation observed for insulin-sensitive subjects at 10-20 minutes. Using specific inhibitors the abnormal enzyme activities were identified as a type-1 phosphatase (PP-1) in human muscle from insulin-resistant subjects. The abnormally low fasting PP-1 activity in insulin-resistant subjects persisted following trypsin treatment, suggesting that inhibitors 1 and 2 (characterized regulators of PP-1) are not important determinants of the abnormal phosphatase activity. All insulin resistant subjects showed Mn activation of trypsin treated PP-1 in the absence of azide. 6 of 10 insulin sensitive subjects, however, required azide in order to see Mn activation of PP-1. The azide appears to reverse the effects of an inhibitor of Mn activation which as been localized in the glycogen-microsomal (GM) subcellular fraction of Mn-resistant (insulin sensitive) subjects. These results suggest that an azide sensitive structure in the GM fraction of muscle is responsible for the abnormal PP-1 activity in insulin resistant subjects. Phosphorylation of the G-subunit bound to the PP-1 catalytic subunit in the GM fraction regulates PP-1 activity. Western blots of muscle extracts indicate that the G-subunit has increased immuno-reactivity in insulin resistant subjects. In addition, insulin resistant subjects have increased binding of their PP-1 gamma isoform to the GM fraction. Taken together these results suggest that abnormal PP-1 activity in insulin resistant subjects may directly involve the G-subunit.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69021-12

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Energy Expenditure in Pima Indians: Risk Factors for Body Weight

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E. Ravussin	Visiting Scientist	CDNS, NIDDK
Others:	M. Spraul	Visiting Associate	CDNS, NIDDK
	R. Norman	Senior Staff Fellow	CDNS, NIDDK
	C. Bogardus	Chief	CDNS, NIDDK
	J. Young	Professor	Northwestern
	R. Leibel	Professor	Rockefeller Univ.
	M. Goran	Staff Fellow	Univ. of Vermont
	J. Skinner	Professor	Arizona St. Univ.

COOPERATING UNITS (if any)

Indian Health Service; Dept. of Medicine, Northwestern Medical School, Chicago, IL, (J. Young); Rockefeller University, New York, NY (R. Leibel); University of VT, Burlington, VT (M. Goran); Arizona

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.55	2.55	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The prevalence of obesity in the Pima Indian population is extremely high. Since obesity represents a major risk factor for insulin resistance and the development of NIDDM we have focused our effort in seaching for the possible causes of obesity in this population including metabolic and genetic factors. We have identified four known familial metabolic parameters predicting body weight gain: a low metabolic rate, a high respiratory quotient, insulin sensitivity, and a low spontaneous physical activity. Also Pima Indians have a low level of sympathetic nervous activity. Recent data suggest that Pima Indians as a population have a low level of physical activity even at age five. We know that regarding the causes of the variability of these metabolic parameters: 1) age is not a major determinant of sedentary energy expenditure, but is associated with lowering of physical activity in free-living conditions; 2) metabolic rate is regulated to maintain a genetically determined body temperature but Pima Indians have lower sleeping body temperatures than Caucasians; and 3) Pima Indians have a lower sympathetic nervous system activity than Caucasians, and a blunted increase in response to an oral glucose load despite larger increases in plasma insulin levels. This blunted response may represent another feature of the obesity/insulin resistance syndrome. Since a low fat oxidation is a risk factor for body weight gain, we are studying the mechanisms regulating fat oxidation. Skeletal muscle lipoprotein lipase activity and muscle fiber types account for part of the inter-individual variability in energy metabolism. Surprisingly we could not find any relationship between sympathetic nervous activity and substrate oxidation has been found. Preliminary data in a Pima Indian population in nothern Mexico indicate that despite a potential genetic predisposition, a traditional lifstyle may protect against the development of obesity and NIDDM.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69024-07

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

WHO Collaborating Center for Epidemiologic and Clinical

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P.H. Bennett	Chief	PECR, NIDDK
Others:	W.C. Knowler	Chief	DAES, NIDDK
	C. Bogardus	Chief	CDNS, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK

COOPERATING UNITS (if any)

World Health Organization, Non-Communicable Diseases Program, Geneva, Switzerland, (Foreign), World Health Organization Collaborating Centers for Diabetes (Foreign), China-Japanese Friendship Hospital,

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.6	0.1	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Phoenix Epidemiology and Clinical Research Branch was designated as the WHO Collaborating Center for Design, Methodology and Analysis of Epidemiological and Clinical Investigations in Diabetes in 1986. The purposes of the Center are to collaborate with the World Health Organization in the implementation of the WHO/IDF action program to provide advice, consultation and collaboration with other investigators in the design, methodology and analysis of epidemiology and clinical diabetes (NIDDM) and its complications. The center will assist in the development and application of standardized methods for epidemiological and clinical investigations and data analysis relating to the etiology and pathogenesis of non-insulin-dependent diabetes (NIDDM) and its complications. The center will collaborate with those interested in applying such techniques elsewhere. The Center will advise and help in the design of new studies, including on site assistance when necessary. The Center serves to train investigators from many parts of the world in diabetes epidemiology and clinical research.

The Center participates in the WHO Multicenter Study of Vascular Disease in Diabetes, which is examining the mortality and incidence of vascular complications of diabetes among different ethnic groups in different countries.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69025-07

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Treatment of Impaired Glucose Tolerance in Malmöhus County Sweden

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler Chief DAES, NIDDK

COOPERATING UNITS (if any)

Lund University, Dalby, Sweden (Foreign)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The prognosis and effect of treatment of impaired glucose tolerance (IGT) are studied in residents of Malmöhus County, Sweden. Mortality rates are determined in over 2000 persons who had glucose tolerance tests in the 1960s, and results are related to glucose tolerance and other factors at baseline. Some of these subjects participated in a randomized treatment study of IGT. New studies in population screening and treatment of IGT are being planned.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69028-05

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetics of Non-Insulin-Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	P.H. Bennett	Chief	PECRB, NIDDK
	C. Bogardus	Chief	CDNS, NIDDK
	S. Lillioja	Visiting Scientist	CDNS, NIDDK
	M. Prochazka	Senior Staff Fellow	CDNS, NIDDK

COOPERATING UNITS (if any)

Bowman-Gray Medical School, Winston-Salem, NC; Howard Hughes Medical Institute, University of Chicago, Chicago IL; Arizona State University, Tempe, AZ.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.8	0.3	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Non-insulin dependent diabetes mellitus is a common chronic disease that develops in most populations in late middle age. The Pima Indians of Arizona have the highest reported prevalence of this disease in the world and in contrast to many populations the disease often presents at an earlier age. As a result of long-term epidemiological studies in the total population, the familial nature of the disease has been well documented, and segregation analyses suggest the possibility of inheritance by a single additive major gene.

This project will search for genetic determinants of NIDDM using the techniques of genetic linkage analysis with genetic markers to identify the chromosomal location of inherited determinants of NIDDM in the Pima Indian population. A number of informative pedigrees have been identified and lymphoblast cell lines from informative members of these pedigrees established. DNA from these lymphoblasts is isolated and polymorphic probes applied to search for evidence of linkage of these markers and NIDDM. Probes with established chromosomal locations will be used to screen the genome to detect genetic linkage with NIDDM as described in Project# Z01 DK 69045-01 (Molecular genetic analysis of a chromosome 4 region harboring agent controlling insulin action).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 DK 69030-05

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Contribution of Protein Tyrosine Phosphatase to Insulin Resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	M. Kunkel	IRTA	CDNS, NIDDK
	J. Rowles	IRTA	CDNS, NIDDK
	N. Tonks	Senior Staff Fellow	Cold Spring Harbor Scientist Labs., CSH, N.Y.

COOPERATING UNITS (if any)

Indian Health Service; Cold Spring Harbor Labs., CSH, NY (N. Tonks)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.05	2.05	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We previously identified a novel PTPase in human skeletal muscle by PCR and reported the cloning of the full-length cDNA from a HeLa library. We repeated the Northern analysis using a longer probe and found a strongly hybridizing band of ~4.8 kb in rabbit skeletal muscle. We also found two sequencing errors: one was the omission of a 69 bp EcoR1 repeat (causing the predicted protein to have a 23 amino acid "insert"), and the other was the omission of a single G residue (changing the ORF to now encode a protein of predicted molecular weight 88090). This PTPase has been called PTP-PEST due to the presence of four regions rich in Pro, Glu/Asp and Ser/Thr. PEST domains are often found in proteins with short intracellular half lives. In order to determine if the PEST sequences do alter the half life or have any other regulatory role, the full length protein and a truncated version that contains the entire catalytic domain but lacks all the PEST sequences have been expressed in Sf9 cells using a recombinant baculovirus. In order to determine if insulin alters the enzymatic activity or phosphorylation state of this PTPase, each form of PTP-PEST can now be co-expressed with the insulin receptor. In addition, monoclonal antibodies against PTP-PEST are being generated that can be used to determine protein levels and enzymatic activity in skeletal muscle extracts from insulin sensitive and resistant Pimas. Another approach to indentify the PTPases that are present in skeletal muscle is to resolve (and purify) them by chromatographic methods. We have resolved 4-6 PTPases by ion-exchange chromatography from soluble extracts of rabbit skeletal muscle and various muscle-like tissue culture cell lines. The PTPase that elutes at approximately 0.32 M NaCl from anion-exchange resins has been partially purified and has a molecular weight of ~67 kDa and a specific activity of ~10umol/min.mg using an insulin receptor peptide as substrate.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69031-05

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Phosphorylase Phosphatase by Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	B. Thompson	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK
	D. Mott	Supervisory Res. Chem.	CDNS, NIDDK
	S. Norman	IRTA	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.2	1.1	.10

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Impaired activation of glycogen synthase in insulin-resistant subjects is associated with both chronically lower activity of protein phosphatase-1 (PP1) and a smaller magnitude of activation of PP1 in response to insulin compared to that seen in insulin-sensitive subjects. Although PP1 activity is low in muscle of resistant subjects, the concentration of catalytic subunit of PP-1α is higher than in sensitive subjects. The predicted amino acid sequence of the catalytic domain of a 420 bp PP-1α PCR product was examined in insulin sensitive and resistant subjects, and found to be identical. PP1-α RNA concentration and size in skeletal muscle (1.6 kb) were also the same in fasting subjects of different insulin sensitivities. The concentration of RNA corresponding to PP1-α in human skeletal muscle decreased by 50% after 30 min of a high dose infusion of insulin in vivo. The concentration returned to basal levels by 120 min. In contrast, PP1-α RNA increases slightly in response to insulin in insulin-resistant subjects. The cDNA sequence for another PP-1 catalytic subunit isoform from human muscle was found to be 93% similar in coding sequence to PP-1 gamma-1, previously described in rat liver. This isoform was detected by Northern blot in human heart, brain, placenta, lung, liver, skeletal muscle kidney and pancreas. The specific PP-1 gamma-1 sequence was localized to human chromosome 12.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 DK 69036-04

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Epidemiology of Complications of Non-Insulin-Dependent Diabetes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	P.H. Bennett	Chief	PECRB, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	Q.Z. Liu	Visiting Fellow	DAES, NIDDK
	D.R. McCance	Visiting Associate	DAES, NIDDK
	R.L. Hanson	Epidemiology Staff Fellow	DAES, NIDDK
	K.M.V. Narayan	Visiting Scientist	DAES, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Cleveland Clinic Foundation, Phoenix, AZ.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
2.9	2.2	0.7

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of the project is to determine the incidence rates, rates of progression, and risk factors for the chronic complications of NIDDM. The study is conducted in the Pima Indians of the Gila River Indian Community, who have participated in a longitudinal epidemiologic study since 1965 (see project Z01 DK 69000).

Risk factors for the major complications of diabetes, retinopathy, nephropathy, coronary artery disease, and peripheral vascular disease are determined by longitudinal followup of diabetic subjects. Methods of ascertainment of these complications include fundus photography, measurement of urine albumin and serum creatinine concentrations, electrocardiography, and documentation of lower extremity amputations.

Retinopathy and other retinal lesions were assessed by ophthalmoscopy and fundus photography in 288 nondiabetic and 1074 diabetic Pima Indians. Fundus photography was more sensitive in detecting nonproliferative and proliferative retinopathy. Retinopathy was strongly related to the duration of diabetes and to type of diabetes treatment, with the highest prevalence in insulin-treated patients.

The relationships between glycated hemoglobin (HbA1), fasting and two-hour post-load plasma glucose concentrations, and diabetic retinopathy were examined, and the strengths of the associations were directly compared by receiver operating characteristic analysis. All three measures of glycemia were highly associated with each other and with either the prevalence or incidence of retinopathy. HbA1 was slightly, but not statistically significantly, more closely related to diabetic retinopathy than a single plasma glucose determination.

PHS 6040 (Rev. 5/92)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69037-04

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Kidney Function in Non-Insulin-Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett Chief PECRB, NIDDK

Others: W.C. Knowler Chief DAES, NIDDK

COOPERATING UNITS (if any)

Cleveland Clinic Foundation; Stanford University; Emory University; Chronic Renal Diseases Program, NIDDK; Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.7	0.2	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The functional characteristics of the renal glomerulus are being investigated in Pima Indians of the Gila River Indian Community to identify the underlying pathogenetic mechanisms involved in the initiation and progression of renal disease in non-insulin-dependent diabetes mellitus (NIDDM).

The Pima Indian population has a high incidence of NIDDM and diabetic nephropathy. Six groups of subjects are being studied: subjects with normal glucose tolerance, individuals with impaired glucose tolerance (IGT), those with newly diagnosed diabetes (<3 years duration NIDDM), and diabetic subjects (≥5 years duration NIDDM) with evidence of (a.) mildly abnormal albumin excretion (b.) severe abnormalities of albumin excretion (c.) normal albumin excretion. Measurements of renal and glomerular capillary wall function including glomerular filtration rate (GFR), renal plasma flow (RFP), dextran sieving coefficients, and albuminuria are being performed and correlated. Markers and/or predictors of progression as well as the mechanisms of initiation and progression of diabetic renal disease are being sought.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 DK 69039-04

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Dietary Survey of the Pima Indians of the Gila River Indian Community

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	P.H. Bennett	Chief	PECRB, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK

COOPERATING UNITS (if any)

Cleveland Clinic Foundation, Phoenix, Arizona.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

An age-sex-stratified sample of 600 residents of the Gila River Indian Community, ages 18-75 years, was recruited for a dietary survey. Dietary intake was estimated by the dietary history method to obtain quantitative food frequency information. Reported energy intake was higher in men and negatively related to age; thus, relationships with weight were analyzed by multiple regression controlling for sex and age. Energy intake in kilocalories (kcal) was positively associated with body weight or body mass index, adjusted for age and sex. Body weight was associated with absolute intake of the major dietary components, carbohydrate, fat, and protein, but not with any of these components expressed per 1000 kcal, suggesting that total energy intake, rather than proportions of specific components, was the variable having the strongest association with body weight. Alcohol consumption (in any amount) was higher in men and inversely associated with age and weight. Neither energy intake nor specific components were significantly associated with diabetes, after adjustment for age and sex, nor did diabetes affect the relationship between energy and weight, in a subset of 307 subjects (153 diabetics) examined for diabetes within one year of the diet history. The acceptance of obesity in this population may reduce the under-reporting of energy intake which has been postulated in other studies. Body weight in Pima Indians is weakly but positively associated with increased caloric intake.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69040-04

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sodium-Lithium Countertransport and Blood Pressure

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler Chief DAES, NIDDK

Others: D.J. Pettitt Assistant Chief DAES, NIDDK

COOPERATING UNITS (if any)

University of Pittsburgh; University of Southern California

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.1		0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Red blood cell sodium lithium countertransport is a genetic marker of hypertension in several ethnic groups. It reflects the sodium-hydrogen antiport activity in renal tubules. Several recent studies showed an association between sodium-lithium countertransport and predisposition to diabetic nephropathy. We are studying the relationships between sodium-lithium countertransport, nephropathy, and blood pressure in a sample of 200 Pima Indians.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69041-04

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin Resistance in Obesity and the Association with Lymph Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. Lillioja Visiting Scientist CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.25	.25	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Since insulin action in vivo in man is correlated with the density of the capillary supply to skeletal muscle, we hypothesize that since the unfenestrated capillaries of skeletal muscle are relatively impermeable to insulin that the increased insulin resistance in those with low capillary density might be due to altered kinetics of insulin penetration to its sites of action in the muscle. A method was developed to directly collect lymph from a peripheral lymphatic vessel in sufficient amounts to measure insulin, and glucose during changes in arterial insulin concentrations. Insulin concentrations in limb lymph were found to be considerably lower than that in plasma and in contrast to plasma insulin concentrations, lymph concentrations were highly correlated with glucose uptake rates in each individual. In insulin resistant subjects, the slope of the glucose uptake, lymph insulin relationship was much steeper than in lean subjects. Therefore, interstitial insulin concentrations determine insulin action in an individual but the between individual variation in insulin sensitivity is more determined by distal pathways that lead to insulin mediated glucose uptake. This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69043-04

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Gene Expression by Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	B. Thompson	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

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Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.15	.15	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to identify genes that normally respond to insulin in vivo and specifically, to explore the pathway of insulin regulated gene expression in human skeletal muscle in individuals with different insulin sensitivities, we have adapted the S1 nuclease protection assay for use with multiple probes (multiple S1 nuclease protection assay) to allow the simultaneous examination of RNA abundances from multiple genes. In conjunction with hyperinsulinemic, euglycemic clamp technique, we have evaluated the ability of insulin to alter the RNA abundances of several proto-oncogenes and on other potentially insulin responsive genes in human skeletal muscle from individuals with differing insulin sensitivities over a two hour time period. Of the 9 genes examined with this technique, 4 proved to be insulin responsive in vivo in insulin sensitive individuals. The proto-oncogenes c-Ha-ras, c-myc, and c-src all displayed a 2-4 fold transient increases in their respective RNA levels within 30 minutes of insulin infusion. In addition, myf-5, a muscle specific differentiation factor also proved to be insulin sensitive. The abundance of this RNA also increased 3-fold with a time course similar to those displayed by c-Ha-ras and c-src. The responses of c-Ha-ras and myf5 were diminished in insulin resistant individuals. C-myc, while responding to insulin stimulation in both groups showed overall lower levels in individuals with decreased insulin sensitivity. In contrast, c-src RNA levels increased in response to insulin in both groups. While RNA abundance of c-jun and insulin receptor were not altered over the two hour time course of insulin administration, the basal RNA levels were lower in individuals that are insulin resistant. This suggests that there are multiple insulin signal pathways that result in modifications in gene expression. Glut-3, Glut-4 and c-fos showed neither statistically significant increases in RNA levels nor were basal RNA levels altered by decreased insulin sensitivity. This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69044-03

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin Resistance in Obesity and the Association with Membrane

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. Lillioja	Visiting Scientist	CDNS, NIDDK
Others:	L. Storlein	Senior Investigator	Royal Prince Alfred Hospital, Sydney, Australia

COOPERATING UNITS (if any)

Indian Health Service; Department of Endocrinology, Royal Prince Alfred Hospital, Sydney, Australia

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Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.25	.25	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous studies in experimental animals, and a more recent study in man, have indicated that fasting plasma insulin concentrations, as an estimate of insulin action in vivo, are well correlated with muscle phospholipid polyunsaturated fatty acid content. This offers a potential mechanism for the development of insulin resistance with the development of obesity. It is suggested that alterations in the fatty acid content of skeletal muscle phospholipids may change with increasing body fat content and there also affect insulin action. In this study we will examine the relationship of obesity, insulin action in vivo and skeletal muscle membrane phospholipid content in Pima Indians to determine if obesity causes insulin resistance by altering membrane phospholipid content. This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69045-02

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analysis of a Chromosome 4 Region Harboring a Gene Controlling

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. Prochazka	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK
	L. Baier	IRTA	CDNS, NIDDK
	J. Sacchettini	Associate Professor	Albert Einstein College of Med
	J. Tait	Associate Professor	Univ. Washington

COOPERATING UNITS (if any)

Indian Health Service; Department of Laboratory Medicine, University of Washington, Seattle, WA.; Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY.

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Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.15	.15	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our recent linkage study in Pima Indians provided evidence for a gene determining insulin action located near two markers on chromosome 4q, the intestinal fatty acid binding protein gene (FABP2), and the annexin V gene (ANX5). Extensive typing of numerous polymorphic markers on 4q narrowed this putative gene to a chromosomal segment about 5 centiMorgan long (an estimated $5x10^6$ base pairs). Because FABP2 and ANX5 are the only well-positioned functional genes in this region, two strategies are being pursued to search for the putative gene. First, we are exploring the FABP2 gene itself as a potential candidate because of the known role of fatty acids in insulin action. Analysis of FABP2 coding sequences by the single-strand conformation polymorphism technique (SSCP) detected a single base substitution (G --> A) in the second exon resulting in an Ala to Thr substitution at position 54 of the protein. The structural and functional significance of this substitution is currently under investigation. Second, we have initiated a search for new genes in the FABP2/ANX5 region by using the yeast artificial chromosome (YAC) genomic cloning system. Molecular analysis of a YAC clone carrying the ANX5 gene led to the discovery that the cyclin A gene (CCNA), previously assigned to 4q by in situ hybridization, is located within 150 kilobases from ANX5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69046-02

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analysis of Chromosome 19 in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. Prochazka	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK
	M. Majer	Special Volunteer	CDNS, NIDDK
	H. Mochizuki	Special Volunteer	CDNS, NIDDK
	D. Robertson	Visiting Scientist	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

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Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.15	.15	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Current evidence indicates that multiple genes contribute to the familial predisposition for NIDDM in Pima Indians. To search for such genes, we have undertaken a "candidate gene" approach investigating loci which code for proteins involved in the control of glucose homeostasis. Simultaneously, a random approach was initiated using highly informative chromosomal linkage markers. Our group have chosen chromosome 19 because it carries at least three candidate genes including the insulin receptor gene (INSR), the glycogen synthase gene (GYS), and the gene for a hormone-sensitive lipoprotein lipase (LIPE). The latter gene was selected because of the high prevalence of obesity in Pima Indians which increases the risk for NIDDM. Recently, the myotonic dytrophy gene (DM) on this chromosome was also included as a potential candidate because of the documented insulin resistance in patients affected by this muscular disorder. Highly polymorphic DNA markers within each of these genes were utilized in our study. To cover the rest of chromosome 19, nine additional informative DNA markers (D19S177, D19S76, D19S204, D19S179, D19S47, APOC2, KLK1, HRC, D19S246) were chosen for typing. Each marker is being analyzed for linkage with several phenotypes in Pima Indians including NIDDM, insulin resistance, and obesity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69047-02

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mapping Chromosome 11 in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	B. Thompson	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.20	.20	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Chromosome 11 contains several candidate genes that may be involved in the etiology of insulin resistance and type II diabetes. These candidate genes include the insulin gene, protein phosphatase 1 alpha, glycogen phosphorylase, c-Ha-ras, and IGF-2. In an effort to search for chromosomal regions and subsequently the genes involved in insulin resistance and type II diabetes polymorphic markers are being used to examine chromosome 11 at a 10 centimorgan resolution.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69048-02

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Identification of Insulin Regulated Transcription Factors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:B. Thompson Senior Staff Fellow CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.25	.25	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous work on insulin regulation of gene activity revealed that insulin-resistance alters insulin regulated gene expression. Insulin-resistance alters immediate early gene expression in a gene specific manner, suggesting multiple insulin signal pathways that result in changes in gene expression. Two genes, c-Ha-ras and myf5, displayed the same types of insulin mediated responses in insulin-sensitive and -resistant individuals suggesting the same mode of regulation. To isolate the cis-acting sequences and the trans-acting factors that bind these sequences that are responsible for the changes in RNA levels for these two genes, genomic clones of myf5 have been isolated. The 5' regulatory region will be analyzed for insulin responsive elements. Once isolated the insulin responsive DNA element can be utilized to test insulin-sensitive and -resistant skeletal muscle for differences in the trans-acting factor that interacts with this element.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69049-02

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

SSCP Analysis of PP-1 Alpha and Gamma in Relationship to Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	B. Thompson	Senior Staff Fellow	CDNS, NIDDK
	D. Mott	Supervisory Res. Chem.	CDNS, NIDDK
	S. Norman	IRTA	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

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Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.30	.20	.10

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous studies on the type-1 serine/threonine protein phosphatase (PP-1) activity in skeletal muscle from fasted subjects indicated increased amounts of activity in insulin-sensitive individuals, but increased protein amounts in insulin-resistant subjects. New isoforms of PP-1 have been cloned from muscle cDNA suggesting that PP-1 activity may be a composite of several proteins. Mutations in the coding regions of the skeletal muscle PP-1 isoforms could explain the activity and enzyme content differences. Currently, the genes for the skeletal muscle protein phosphatase isoforms are being characterized. The genomic clones for these genes are being analyzed and suitable PCR primer pairs chosen from the non-coding regions that will allow amplification of PP-1 coding regions. The coding regions of these genes are being examined in insulin-sensitive and -resistant persons for single stranded conformational polymorphisms (SSCP). SSCPs that relate to amino acid changes will be further tested for a relationship to insulin resistance and type II diabetes. At this time SSCP analysis has been completed on 40 subjects for 6 exons from PP-1 gamma-1. This covers 95% of the coding sequence. No sequence abnormalities were observed which were uniquely associated with insulin resistance.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69050-02

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Aspects of the Acute Insulin Response in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Thompson Senior Staff Fellow CDNS, NIDDK

Others: C. Bogardus Chief CDNS, NIDDK

R. Janssen Graduate Student Arizona St. Univ.

COOPERATING UNITS (if any)

Indian Health Service; Arizona State University

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.25	.25	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous observations of variations in the acute insulin response suggested that this phenotype was controlled by one or more genes. Glucokinase, which has been linked to the MODY (maturity onset diabetes of the young) form of diabetes, has been proposed as an intracellular glucose sensor and appeared to be a likely candidate gene. Similarly, GLUT2, the major glucose transporter of the pancreas could also be responsible for the variation observed in the acute insulin response. Polymorphisms at both of these genes have been examined for linkage with the acute insulin response. In an analysis of 117 sibpairs linkage between this region on chromosome 3 and the acute insulin response could not be excluded. A structural analysis of the coding regions for the gene encoding GLUT2 revealed a single transition which predicts an amino acid substitution at position 110 of GLUT2. It is currently unknown how the mutation effects glucose sensing in the ß-cell.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69051-01

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Prevention of Non-Insulin-Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	K.M.V. Narayan	Visiting Scientist	DAES, NIDDK
	R.L. Hanson	Epidemiology Staff Fellow	DAES, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	P.H. Bennett	Chief	PECRB, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Cleveland Clinic, Phoenix, AZ; University of Pittsburgh.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.8		0.6

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Prevention strategies will be developed to take advantage of the metabolic and behavioral risk factors for NIDDM which have been identified in research with the Pima Indians and elsewhere. Obesity, impaired glucose tolerance, hyperinsulinemia, physical inactivity, and high fat diet have been implicated as risk factors for NIDDM. Since these factors are potentially reversible with behavioral and pharmacologic therapy, strategies for prevention will be developed and tested. A feasibility study of diet and exercise treatment has been planned and initiated among overweight Pima Indians with normal glucose tolerance. Knowledge and experience gained from this pilot study will be used in planning and implementing future clinical trials in diabetes prevention.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 DK 69052-01

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of SH2-Domain PTPase by Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. Sell IRTA CDNS, NIDDK

Others: A. Ullrich Max Planck Institute
Martinsried, Germany

COOPERATING UNITS (if any)

Indian Health Service; Department of Biology, Max-Planck Institu, Martinsried, Germany

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
.5		.5

CHECK APPROPRIATE BOX(ES)

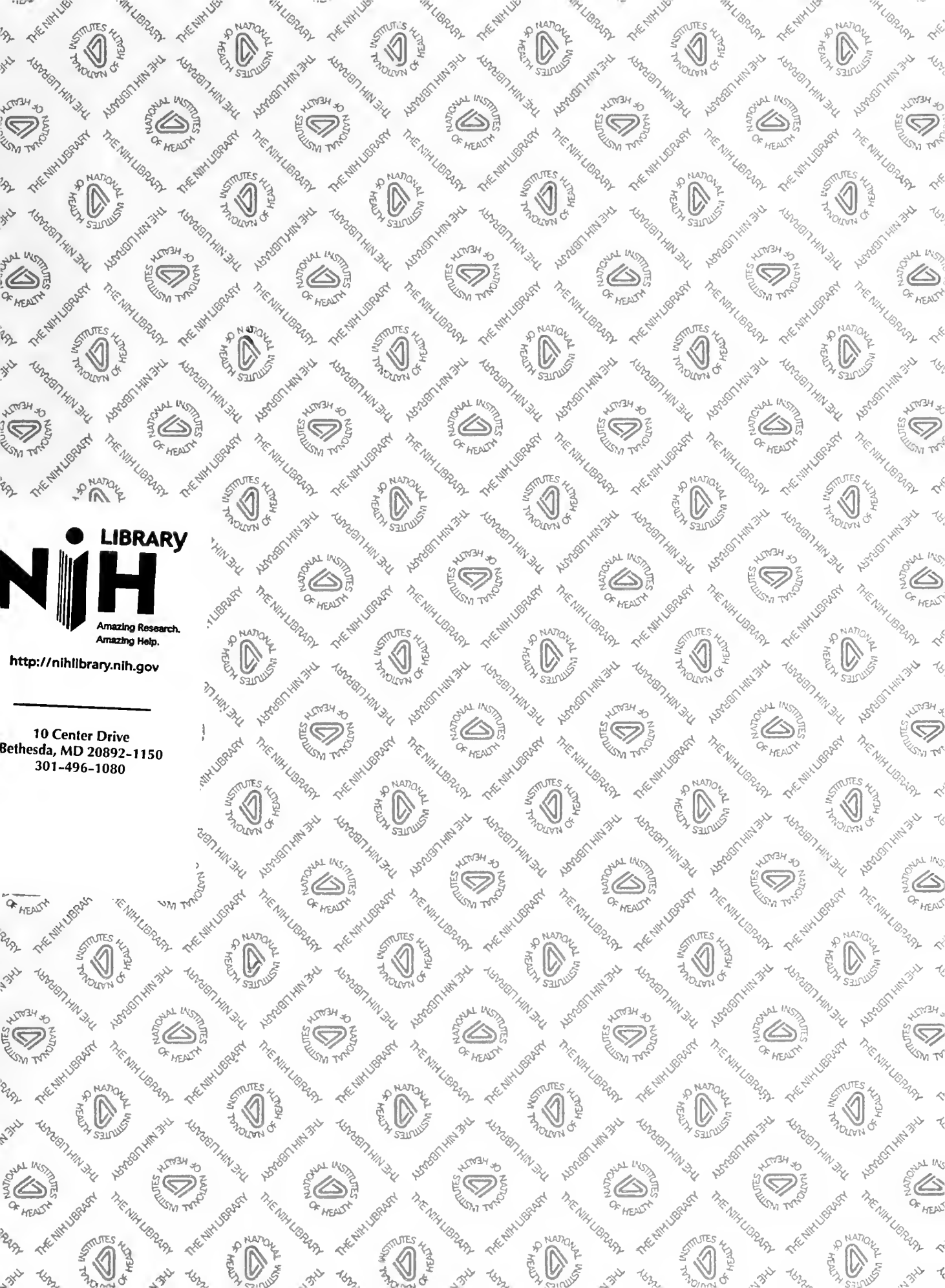
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The abnormal regulation of insulin signalling in insulin-resistant subjects is associated with inadequate suppression of soluble protein tyrosine phosphatase (PTPase) activity in skeletal muscle (Sommercorn, CDNS, NIDDK). Multiple PTPases are likely to be involved in the signalling cascade. SH2-Domain PTPases are a class of PTPases selected as candidates for regulating tyrosine phosphorylation following insulin stimulation since it has been reported that such PTPases associate with several receptors and the post-receptor target, IRS1. SH2-Domain PTPase expression was detected in FAO cells, a rat insulin-sensitive hepatoma cell line, and in C2C12 cells, a mouse muscle cell line, using RT-PCR and degenerate primers (Sell, CDNS, NIDDK). These cell lines represent the two insulin-sensitive tissues reported to exhibit a drop in soluble PTPase activity following insulin stimulation: hepatic and skeletal muscle tissues. Sequence analysis of the amplified products revealed a high degree of identity with the hematopoietic SH2-DOomain PTPase, PTP1C. A related SH2-Domain PTPase, PTP1D, has since been reported to be expressed in liver and skeletal muscle. Experiments will be conducted in the future to characterize the regulation of SH2-Domain PTPases in insulin-responsive cells (FAO and C2C12) and skeletal muscle by insulin. RNA levels will be compared in insulin-resistant and sensitive subjects. Antibodies, specific for PTP1C and PTP1D and generously provided by the laboratory of Dr. Axel Ullrich (Max-Planck), will be used to compare protein levels. Basal levels as well as levels following insulin stimulation will be compared. The predicted amino acid sequence will also be compared in Pima subjects.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